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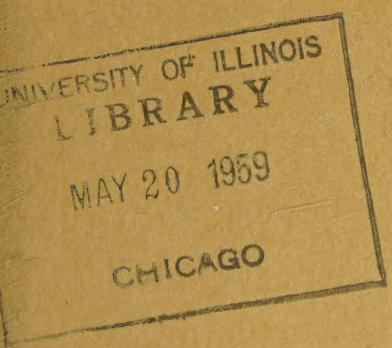

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THE EFFECT OF CHLORAMBUCIL (CB 1348) ON GROWTH AND METABOLISM [1]

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FIVE FIGURES

One of the nitrogen mustard derivatives that is presently being used as a carcinostatic agent in treatment of various malignant lymphomas is p-(di-2-chloroethylamino)-phenylbutyric acid (Chlorambucil) (Galton et al., '55). Delga ('52) reported strong inhibition of yeast growth by two of the nitrogen mustards but only slight inhibition of respiration. However, there have been conflicting reports (Hutchens and Podolsky, '54; Podolsky and Hutchens, '54) in which both respiration and growth have been affected. In this work an attempt was made to study the effect of one of the nitrogen mustard derivatives on yeast metabolism, to substantiate these effects with a mammalian organism (rat) and ultimately to suggest a mechanism of action to correlate these data with the carcinostatic effect of the drug.

METHODS

The yeast used was *Candida utilis,* grown on media composed of 1.2 gm ammonium chloride, 0.5 gm potassium phosphate (monobasic), 0.1 gm calcium chloride, 0.2 gm magnesium chloride and 0.2 gm of yeast extract per liter of distilled water. Two per cent solutions of glucose, sucrose, fructose, pyruvate, citrate, acetate or succinate, in the above salt solution, were

[1] This work was supported in part by a transfer of funds agreement with the National Cancer Institute.

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used as carbon sources, and in some cases glutamate was used as both carbon and nitrogen source. The yeast was grown at 30°C and the growth experiments were conducted in open Warburg flasks under constant agitation to maintain adequate aeration. Growth rates were determined turbidimetrically using the Baush & Lomb Spectronic 20 and plotting light transmission against time. A series of chlorambucil dilutions ranging from 10^{-3} M to 10^{-4}M was used, with 2×10^{-4} M concentrations being the lowest to give complete inhibition. Respiration rates were measured using standard Warburg techniques (Umbreit et al., '45). In the animal studies 20 three-month-old Osborne-Mendel rats, 10 of each sex, fed on ground Purina Laboratory Chow, were used in each group. Blood glucose determinations were made using the Nelson-Somogyi method (Somogyi, '52). Heparinized glassware was used in drawing blood from the tail vein. Three standards were run in duplicate with each glucose determination.

RESULTS

The growth of yeast in glucose, sucrose, fructose or pyruvate medium was completely inhibited by a 2×10^{-4} M solution of chlorambucil. On the other hand, if citrate, acetate, glutamate, or succinate were used as carbon sources, the degree of inhibition was substantially less (about 50% in a series of 10 experiments). The results of one such experiment are illustrated in figure 1. Although two different concentrations of yeast were used as starting points, additional experiments showed that this yeast concentration had no effect on the results.

Chlorambucil (2×10^{-4} M) completely inhibited respiration on the glucose substrate, but when citrate was used the inhibition was only about 50% (fig. 2). The normal respiratory rate of yeast in the glucose medium was approximately the same as in citrate. The respiratory results, therefore, paralleled the growth data. Chlorambucil also inhibited fermentation of yeast on a glucose medium (fig. 3). It can be seen from this graph that the addition of citrate to the Warburg flask does not reverse the inhibition, showing that the citrate does not

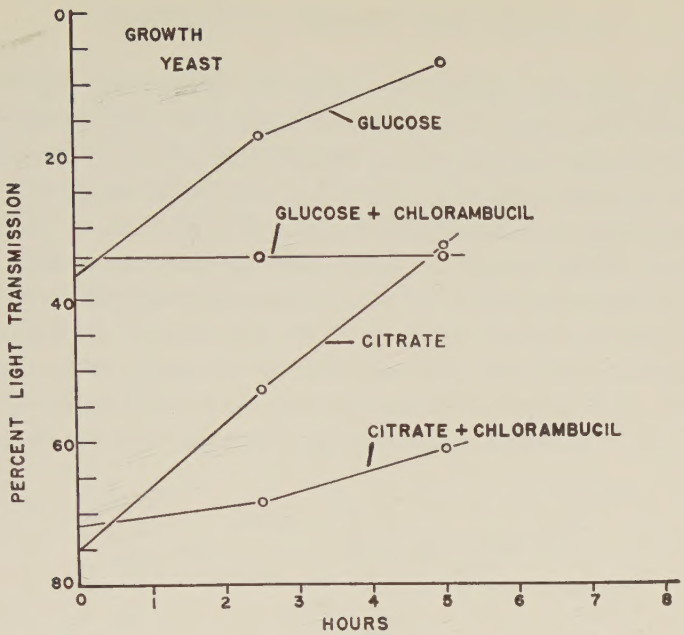

Fig. 1 Growth of yeast *(Candida utilis)* in both glucose and citrate media, with 2×10^{-4} M chlorambucil.

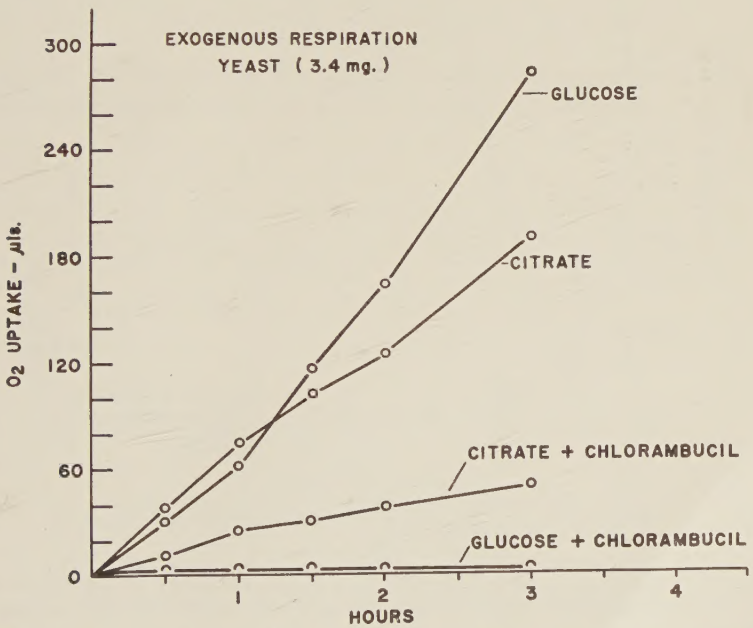

Fig. 2 Exogenous respiration of yeast *(Candida utilis)* in both glucose and citrate media, with 2×10^{-4} M chlorambucil.

prevent complete inhibition simply by complexing with chlorambucil. The order of addition of the citrate and chlorambucil did not change the results. If citrate was added to the medium before the chlorambucil, inhibition was still complete.

According to these data it appeared that glucose utilization was the major point of inhibition by chlorambucil. Blood glucose determinations in rats substantiated this hypothesis. Blood glucose levels (fig. 4) were determined in normal, in chlorambucil-treated (10 mg / kg by stomach tube daily for 7 days), and in pair-fed control rats. As can readily be seen, there was a marked difference in blood glucose levels (deter-

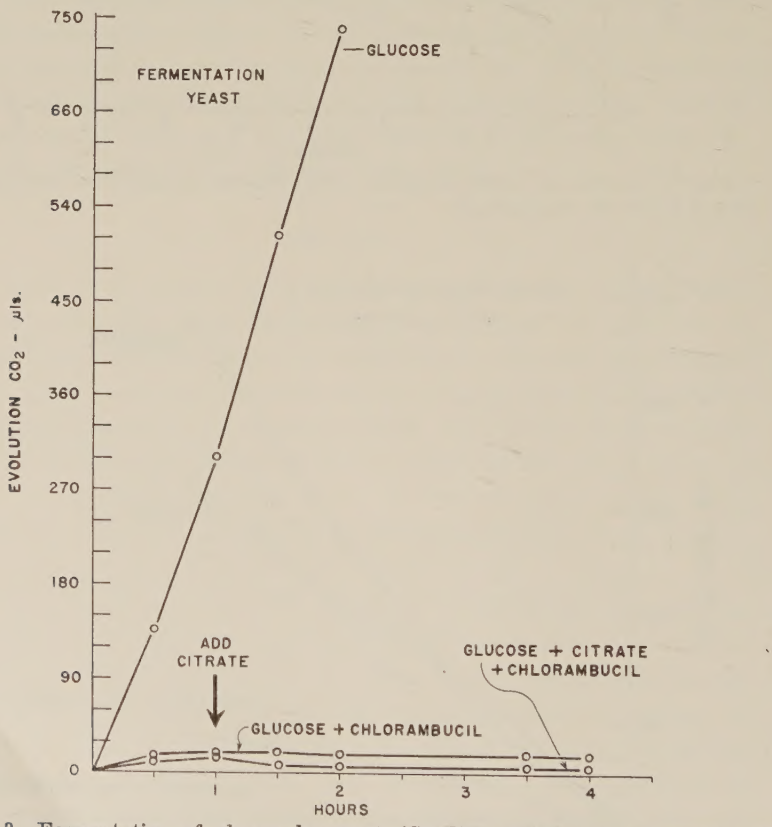

Fig. 3 Fermentation of glucose by yeast *(Candida utilis)* in the presence of 2×10^{-4} M chlorambucil, showing that presence of citrate does not reverse the inhibition.

mined 18 hours after chlorambucil treatment) between the chlorambucil-treated group and its pair-fed control. The chlorambucil-treated group rose to a value above normal and after the 4th administration began to fall to a level just below normal. In contrast, the pair-fed control blood glucose levels fell continually during the course of the experiment. There were no differences due to sex. The food consumption of the drug-treated rats, as shown in figure 5, dropped rapidly and

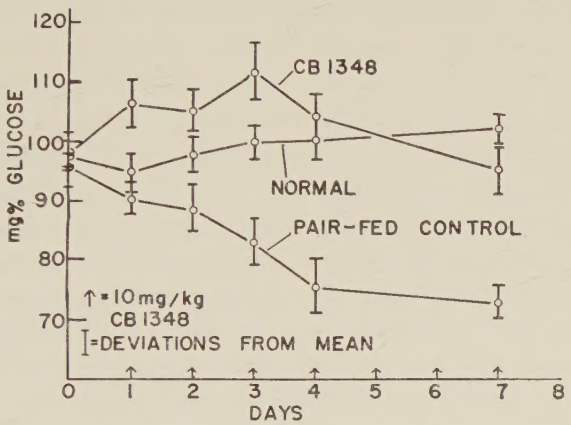

Fig. 4 Blood glucose levels of normal, pair-fed and chlorambucil (CB 1348) treated rats, showing twice the standard deviation of the mean.

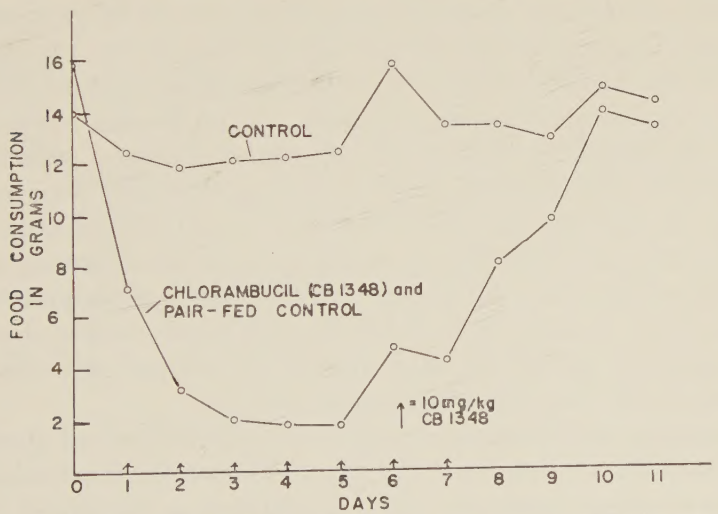

Fig. 5 Food consumption of normal, pair-fed and chlorambucil-treated rats.

remained low until the drug was discontinued. Rats fed subacute doses of chlorambucil for 30 days showed splenic, testicular germinal epithelial, liver and bone marrow atrophy when studied histologically (Hagan et al., '57).

DISCUSSION

The evidence indicates that chlorambucil (2×10^{-4} M) inhibited growth, respiration and fermentation when glucose or pyruvate were used as carbon sources. However, when the Krebs cycle intermediates, citrate, glutamate or succinate, were used, this inhibition was only about 50%. The inhibition due to chlorambucil lasted for about 22 hours, at which time the drug apparently decomposed and growth and respiration rapidly resumed, while the addition of more drug caused a further inhibition. The lower inhibition obtained when using citrate is not simply due to binding of chlorambucil by the citrate. These facts strongly suggest that glucose metabolism is preferentially affected by this carcinostatic drug. This is substantiated by the rat blood glucose determinations, where the feeding of chlorambucil caused the blood glucose level to remain high, while animals fed the same amount of food showed a marked depression of blood glucose. Chanutin and Ludewig ('47) also showed high glucose levels in rats after nitrogen mustard treatment but did not discuss the phenomenon. Dixon and Needham ('46) reported the *in vitro* inhibition of hexokinase by nitrogen mustards, but no *in vivo* work was done. Pathological studies indicate that the tissues primarily affected by chlorambucil (spleen, testis, liver and bone marrow) are rapidly metabolizing tissues, and thus tissues in which one could first expect damage due to interference with glucose utilization. It is conceivable that the carcinostatic effect of this drug could be related to this phenomenon, that is, that glucose utilization is inhibited in tumors affected by chlorambucil, thus inhibiting their growth.

Podolsky and Hutchens reported an inhibition of growth ('54) and respiration ('54) in yeast *(Sacchromyces cerevisiae)* due to nitrogen mustards. They concluded, as do others (Bo-

denstein, '48 and Gilman et al., '46) that this inhibition was due to inhibition of nucleic acid and protein synthesis. This conclusion was based on the fact that in their experiments protein and nucleic acid synthesis fell off markedly upon application of the nitrogen mustard to the medium. However, it seems possible that this inhibition of nucleic acid and protein synthesis could be a secondary effect due to an inhibition of energy metabolism such as glucose utilization, and that the mechanism of action of the nitrogen mustards used by these workers could be the same as that of chlorambucil.

SUMMARY

Chlorambucil (2×10^{-4} M) inhibited both the growth and respiration of yeast grown on a glucose substrate. When citrate was used as a substrate, the inhibition was greatly lessened. Blood glucose levels in rats fed chlorambucil remained quite high as compared to normal and pair-fed rats. In pathological studies of the tissues it has been noted that the effect of chlorambucil was primarily on rapidly metabolizing tissues. A possible correlation between this effect and the carcinostatic effect of this drug is indicated.

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DISTRIBUTION OF STRETCH AND TWIST ALONG THE GROWING ZONE OF THE SPORANGIOPHORE OF PHYCOMYCES AND THE DISTRIBUTION OF RESPONSE TO A PERIODIC ILLUMINATION PROGRAM [1]

R. COHEN [2] AND M. DELBRÜCK
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TEN FIGURES

In previous experiments on the growth response to light in Phycomyces (Delbrück and Reichardt, '56) the entire growing zone (GZ) of the sporangiophore was subjected to stimulation, and the integrated growth output was observed by taking measurements at the sporangium. For a deeper analysis of this response system, as well as for an understanding of the tropic responses and their relation to spiral growth, it is necessary to study the behavior of small portions of the GZ.

The GZ is the organ of growth, the organ of perception of light, and the effector organ executing the growth responses. This organ is in a constant state of turnover and the turnover is of an unusual kind. The organ increases in size by interposition of new material throughout the length of the GZ, but this increase is compensated for by a constant rate of conversion to non-growing stalk taking place at the lower end of the GZ. The balance between new growth and conversion at the lower end is nicely regulated. We should imagine that the sensory elements are distributed throughout the length of the GZ and around its circumference. These are not fixed elements,

[1] Supported by a grant from the National Science Foundation. Technical assistance of Lois Glass Edgar is gratefully acknowledged.

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however. Each of them travels down the GZ and around it, due to stretch and twist. Moreover, we should assume that new elements are created where growth occurs, i.e., throughout the GZ. Moreover, as these receptors evolve there presumably occur changes in their qualities such as their sensitivity, their adaptive properties, and their coupling to the growth processes. All of these properties must be integrated in a well-organized manner to produce the orderly growth and tropic responses. It is this organization which we intend to analyze.

Such an analysis involves a conceptual difficulty. How are we to characterize a particular section of the growing zone? Should we consider *geometrical* sections of fixed position relative to the sporangium? Such a section in the absence of stimulation may have constant properties as a function of time, but the material of which it is constituted will change. The material stimulated at time 0 by a narrow band stimulus impinging upon the section will have travelled out of the section a few minutes later and be replaced by other material which never received the stimulus. Moreover, a geometrical section, by its definition, does not stretch and therefore cannot exhibit a growth response. It is therefore not a concept of practical value. If, on the other hand, we consider *material* sections bounded by markers travelling down with the growth, we are also in difficulty. A material section, even in the absence of stimulation, changes in width and location in the course of time, and even though it may continue to contain the material elements present at the moment of stimulation, it will add new material, which did not receive the stimulus. Moreover, if we want to consider not only light *pulses* in our illumination program, but also stimuli of more complex shape, such stimuli would have to follow the material element in its changes of position and width. Moreover, in the study of growth responses, the standard of comparison is a complex one. For the study of the behavior of the whole GZ, the standard of reference is the speed of growth of the top, which is constant in the absence of stimulation. In the present case, when we consider the properties of the material section, the standard of refer-

ence is the behavior of this section in the absence of stimulation. This standard is variable from location to location within a specimen. It is, therefore, a difficult standard for the experimenter.

We learn from this discussion, on the conceptual side, that a material section of the GZ is not the equivalent, on a reduced scale, of the whole GZ. Its growth response cannot even be formulated in the same terms as that of the whole GZ. The whole GZ is a *constant* structure, in the absence of stimulation, although it is in a state of turnover, like the flame of a candle. Each *material* section of the GZ, however, is not constant: it stretches, twists, and moves down within the GZ. Experimentally, the chief difficulty of defining growth responses of material sections lies in the establishment of standards of reference.

The question of the organization of the GZ may be put in this form: is each material section of the GZ *autonomous* in its responses to light? Does a stimulation of a short section evoke changes in stretch, twist and level of adaptation in the stimulated part only, or is the stimulus transmitted to other parts? We do not yet know the answer to this question. However, *if* each section is autonomous, then it would follow from the above discussion that the sections will *not* react equally with the same time constant of adaptation and the same time course and magnitude of the growth response. This is seen most clearly by considering a section of the GZ, which at the moment of stimulation is located near the base of the GZ. This section will pass out of the GZ before the GZ as a whole has completed its response. Such a section, therefore, would not "have time" to complete a growth reaction in phase with the others.

It is clear that we need to know first of all the behavior of the material sections in the *absence* of stimulation. Except for some minor qualifications to be discussed later, the GZ of a sporangiophore during stage IVb approximates a steady state quite well. The overall growth rate is constant, the rate of rotation of the sporangium due to helical growth is constant, and the length of the GZ is constant. There exist then distribu-

tions of stretch and twist along the GZ which are functions of the distance x from the sporangium, but independent of the time t. It is these two functions which we aim to determine. They characterize the true growth processes at any level x. A similar analysis has previously been performed by Castle ('37). Our experimental procedure gives more detailed data and requires a more detailed mathematical formulation.

The next step is a study of the distribution of the growth *response* and of the spiral growth *response* through the length of the GZ when the whole GZ is stimulated, and this also will be presented.

The responses to stimulations of short sections will be presented in a later paper (Cohen and Delbrück, '59).

Mathematical formulation of the problem

We consider an element in the wall of the sporangiophore and characterize its position by its distance x from the sporangium (with $x = 0$ at the bottom of the sporangium, and increasing downward) and by its azimuth angle α relative to that of a marker on the sporangium or at the top of the growing zone, α increasing for counter clockwise rotation as seen from above (fig. 1).

Growing zone (GZ). A wall element will be considered as part of the GZ if its height above ground increases (due to stretch below it) *and* its distance from the sporangium increases (due to stretch above it). The last part of this definition excludes from the GZ the uppermost part of the stalk, about 0.12 mm long, which does not stretch appreciably.

Primary data. In our measurements we characterize a wall element by a small marker (a starch grain). We attach such a marker to an element very near the upper end of the growing zone when its $x = x_0 = 0.12$ mm. The speed of the marker, dx/dt is necessarily positive and steadily increasing. In the course of 3–4 hours the marker travels through the growing zone. During all this time, at very short time intervals both x and α are measured. These two functions, therefore, $x = f(t)$

and $\alpha = a(t)$, constitute our primary data. From these we wish to extract the two functions $S(x)$, the distribution of the intensity of stretch, and $T(x)$, the distribution of the intensity of twist.

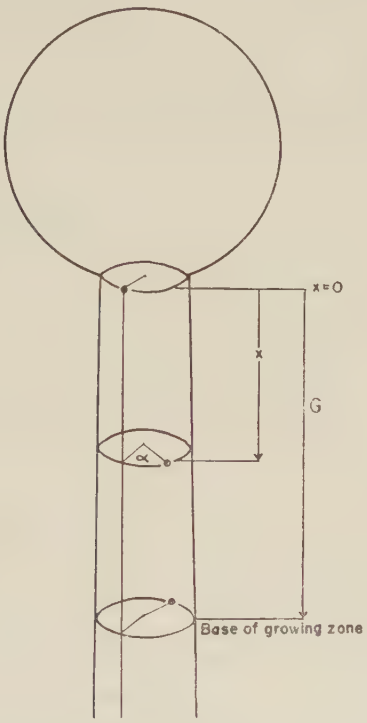

Fig. 1 Definition of vertical coordinate x and of azimuth angle α, both relative to a marker above the base of the GZ. G is the distance to the base of the GZ from the sporangium.

Stretch distribution. The time derivative of x, $v(t) = dx/dt$, describes the speed of a particular marker, relative to the sporangium, as a function of time. This speed may also be looked upon, and plotted, as a function of x. As such, it is not only characteristic for the particular marker, but is, in the steady state, the same function for all markers. It is the *distribution of speed* of the wall elements in the growing zone. We will call this distribution function of speed $u(x)$. We now

define the stretch as follows. Let us consider a very short element of the GZ. Its length Dx will increase with time due to stretch within this element. During a short time its increase d Dx will be proportional to its length and to the *intensity of stretch.* Thus, the stretch as a function of time is given by

$$S(t) = \frac{1}{Dx}\frac{d\,Dx}{dt}. \qquad (1)$$

The intensity of stretch, so defined, is distributed as a function of x according to

$$S(x) = du/dx. \qquad (2)$$

Indeed, consider the upper edge of the element Dx, located at x, and its lower edge, located at $x + Dx$. The upper edge moves with the velocity $u(x)$ and the lower edge with the velocity $u(x + Dx) = u(x) + Dx\ du/dx$. Per unit of time, the distance between the upper and lower edge, therefore, increases by Dx du/dx, from which follows directly the relation given above. S has the dimension time^{-1}. It is the fractional change in length per unit of time of an element located at x.

Twist distribution. As the marker moves down, its azimuth, α, changes. The rate of change of α with time is the angular speed. As in the case of the vertical coordinate x this angular speed is first determined as the time derivative of α, $d\alpha/dt = b(t)$ for the particular marker. It is then considered, and plotted, as a function of x, and then represents *the angular speed distribution function,* $w(x)$, valid for any marker. We define the twist distribution as follows. Consider a short section of the growing zone of length Dx, its upper edge located at x, its lower edge located at $x + Dx$. The upper edge will rotate with the speed $w(x)$, the lower edge with the speed $w(x + Dx) = w(x) + Dx\ dw/dx$. The twist of this element, per unit of time, is therefore Dx dw/dx, and the twist per unit of time and per unit of length, as a function of x, is given by

$$T(x) = dw/dx. \qquad (3)$$

It is the change in angular speed per unit of x and has the dimension angle time^{-1} length^{-1}.

The two functions S(x) and T(x) constitute the local characteristics of the growth. The determination of these functions by experiment is necessarily limited to regions in which the linear and angular speed have reached measureable values. One may inquire, however, whether the two functions can be extrapolated back in some reasonable way to an ideal starting line. Let us imagine that we follow a marker *backward* in time until it reaches speed zero at this starting line. It will be shown that it necessarily takes an infinite time to go back to this starting line *if the stretch function remains finite.* Indeed, since the speed is given by the integral of the stretch function (see equation 2), it starts out from zero with a finite or zero slope. Near the origin, therefore, u(x) may be approximated by a power series, starting with the first or a higher power. The time of travel between any two points is given by an integral involving the reciprocal of the speed

$$t_{1,2} = \int_{x_1}^{x_2} dx/u(x). \qquad (4)$$

This integral necessarily diverges at the origin when u(x) has the indicated behavior.

The travel time of a marker from the ideal starting line to any particular position in the GZ is therefore infinite. In the real situation, of course, an infinite travel time is meaningless. We conclude, then, that the idealization of a perfect steady state is inappropriate for a description of the events near the upper boundary of the GZ.

The steady state approximation, is, however, suitable for the calculation of travel times of a marker from the actual experimental starting line, where the speed has a *small finite value* u_0, to any position x, and particularly for the calculation of the total travel time needed to reach the lower end of the GZ.

The travel time function, t(x), is obtained by two integrations. First, we obtain the speed as a function of position

$$u(x) = \int_{x_i}^{x} S(x)dx \ , \qquad (5)$$

x_i being the ideal starting line. In this integral we may safely

take the ideal starting line as the lower limit of the integration interval.

The second integration is given by equation (4), with the experimental starting line x_0 for the lower limit of integration. It is instructive to carry out these integrations with a model function $S(x)$. Let us assume that $S(x) = s = \text{constant}$, between the ideal starting line x_i, and the bottom of the GZ at G. The value of this constant is fixed when the final speed V is given:

$$V = u(G) = s(G - x_i) \quad \text{or} \quad s = V/(G - X_i) \tag{6}$$

(If the stretch function is not constant, the ratio $V/(G - x_i)$ is its average value.) The first integration now gives a linear speed distribution

$$u(x) = u_0 + s(x - x_0) \tag{7}$$

and the second integration gives

$$t(x) = (1/s)\log\,[u(x)/u_0]\,. \tag{8}$$

The travel time, therefore, in this model increases as a logarithmic function of u. If we insert values corresponding to a representative real case

$$(s = 0.025\ \text{min}^{-1},\ u_0/u_G = 0.01),$$

we obtain a total travel time around 350 min. Actual travel times are about half as long due to the fact that the real stretch functions are not uniform, but have a peak near the origin.

We now enquire into the relations between twist and stretch, between linear and angular velocities, and between vertical position and azimuth angle. Given the twist function, $T(x)$, we have to deal again with two integrations. The first gives the distribution of angular velocity

$$w(x) = \int_{x_i}^{x} T(x)\ dx. \tag{9}$$

It is mathematically and physically reasonable to assume that $T(x)$ remains finite at the ideal starting line, and that the angular velocity therefore starts out from zero or finite slope. Indeed, this is compatible with what is found experimentally; near the experimental origin the angular speed does not vary

violently as a function of the vertical coordinate. The second integration gives the azimuth angle α as a function of vertical position.

$$\alpha(x) = \int_{x_1}^{x} (d\alpha/dx)\, dx = \int_{x_1}^{x} (d\alpha/dt)\cdot(dt/dx)\, dx = \int_{x_1}^{x} [w(x)/u(x)]\, dx. \qquad (10)$$

In this integral we do not have to worry about a singularity at the ideal starting line; although both numerator and denominator in this integral go to zero at the origin, their ratio seems to approach a finite value.

Of particular interest is the model case where stretch and twist are everywhere proportional to each other. In this case it is easily seen that angular and linear speed are also proportional to each other, and so are the azimuth angle and vertical coordinate. Our experiments show certain characteristic differences between this model case and the real situation.

MATERIAL AND METHODS

The methods for culturing the material and the illumination equipment have been described previously (Delbrück and Reichardt, '56). The illumination is bilateral, the two beams reaching the specimens from directions making angles of 60° with the vertical. The equilibrium direction of growth is vertical, and hunting around this direction is minimal.[3] The conditioning light is filtered through Corning No. 61 filters and has a spectral distribution similar to the action spectrum for phototropic effects. Intensities are expressed on a logarithmic scale with logarithms taken to the base 2. The zero of this scale corresponds to an intensity of 100 ergs/cm^2/sec.

[3] Under bilateral illumination two spontaneous oscillations (''hunting'') can occur. The sporangium oscillates around the vertical and nearly in the vertical plane defined by the two lights. One of the oscillations has a period of about 40 min. and an amplitude which varies strongly with the angle between the two incident beams. The amplitude is usually less than 10° for the angle 120° used in the present experiments. The other oscillation, superimposed on the first, has a period of 5 minutes and a maximum amplitude of 2°. A detailed study of these oscillations has been undertaken by D. Dennison (1958).

In the present experiments intensities in the "normal" range were used. In this range the behavior is independent of the absolute intensity.

Growth rate measurements extending over 24-hour periods were performed by two methods. In the first method the specimen is photographed in red light against a dark background at intervals of a few minutes *without moving the film between exposures.* Exposures are so adjusted that only the

Figure 2 a

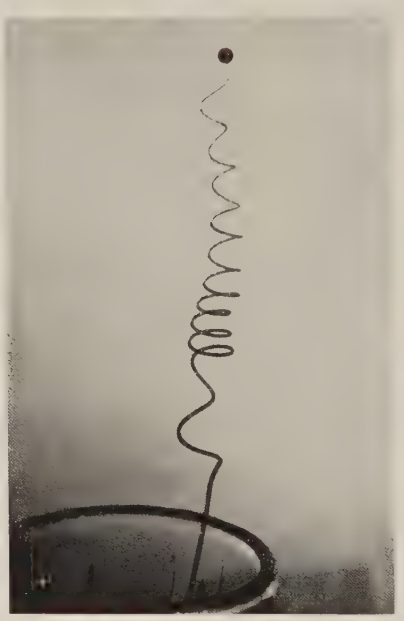

Figure 2 b

Fig. 2 Growth rate measurements extending over 24 hour periods. (a) The specimen is photographed with intense red light at intervals of 15 minutes, without moving the film between exposures. Duration of each exposure 5 seconds. Only the highlight reflected from the sporangium is recorded. In addition, the specimen is illuminated continuously bilaterally with blue light. Its reflection is too weak to score. (b) The specimen is illuminated unilaterally with blue light and is mounted on a turntable rotating with a period of 120 min. Due to phototropism it grows in the form of a helix, with one gyre per rotation. The length of each gyre serves to determine the average growth rate during the particular 120-minute period.

highlight spot of specular reflection from the sporangium is recorded (fig. 2a). The second method consists of placing the specimen on a turntable rotating around a vertical axis with a period of 120 min. and illuminating it from one side. The specimen then grows in the form of a helix of a few millimeters diameter, one turn of the helix representing the growth in a 120-min. interval (fig. 2b). The specimens thus record, in their helix, their average growth speed in successive 120-min. intervals and this can be evaluated micrometrically.

Stretch and twist under steady state conditions. The principal new features in the present experiments consist in the use of markers and in a device for rotating a specimen around its vertical axis. The markers are starch grains of 7 μ diameter, applied to any desired position on the stalk with the help of a micromanipulator. Such markers do not noticeably affect the growth of the specimens. Only when a large clump of markers is applied to the stalk does a slight kink develop at the point of application. This kink travels through the GZ with the clump.

The specimens are placed on a turntable with vertical axis which is rotated at a constant speed, one revolution per minute in most of the present experiments. This rotation requires two adjustments. First, the axis of rotation of the turntable has to be exactly in focus and in the center of the field of observation and secondly, the specimen has to be placed on the turntable in such a manner that the growing zone coincides exactly with the axis of rotation. The first of these adjustments is brought about by attaching the turntable to a micromanipulator capable of fine motions in three directions. The turntable is driven by a small synchronous motor resting on a support on which the motor can slide smoothly in the horizontal directions. The motor is coupled to the turntable through a shaft which slides smoothly in the vertical direction inside the shaft of the turntable. The adjustment of the specimens on the turntable is brought about by placing them on a platform sitting on the turntable. This platform is movable relative to the turntable in the horizontal directions by two

fine screws. This adjustment can be performed without disturbing the motion of the turntable.

The measurements of the coordinates of the marker are made at the moment of its proximal transit. Its *vertical coordinate* is measured either by an ocular micrometer, or by bringing it to a standard reference mark in the field with the help of the micromanipulator operating the turntable, and reading the position of the micromanipulator on a divided circle attached to the screw controlling its vertical movement. The *azimuth angle* of the marker is determined by timing the proximal transit with a stopwatch. This transit can be clocked with an accuracy of $\pm$ 0.4 sec. ($\pm$ 2.5 degrees of angle) by reference to the bright line produced by the dioptric properties of the stalk. If the marker is in the lower, non-growing region of the sporangiophore it will appear at its transit exactly every minute. If the marker is in the GZ it is, in addition, subject to the plant's internal twist. The time elapsed between two successive transits will differ from one minute by an amount depending on the relative directions of the motor and of the internal twist, and on the intensity of the latter. For example, if a transit occurs 59 sec. after the preceding one, the twist has been in the direction of the motor rotation and the marker has turned by 6° relative to the turntable between the two transits.

Distribution of the growth response and of the spiral growth response. The specimen is subjected to periodic stimulation, every 5 minutes a short bilateral stimulus being given, and in the intervals it is under bilateral illumination of constant intensity controlling its level of adaptation. Under these conditions the growth rate and the angular velocity, both measured at the sporangium, vary periodically with a period of 5 minutes. This is the integrated result of periodic variation of the stretch and of the twist in all reacting parts of the GZ. As described above, we ascertain the contributions of the various parts of the GZ to the total response by following the speed and the angular velocity of a marker as it moves through the GZ during the periodic stimulation program. During a single

cycle of 5 minutes such a marker changes its distance from the sporangium only slightly and the data obtained during a single cycle can be referred to a single x value.

Since the specimen twists as it grows, the marker has to be brought back into a standard perspective (proximal transit) for each measurement. For this purpose the specimen is placed on the turntable, centered as above and compensatory rotations are done by hand. The marker height is then read on the micrometer scale and its azimuth read on a protractor coupled to the turntable.

EXPERIMENTAL RESULTS

Growth rate

Errera (1884) designated as stage IV the growing stage after formation of the sporangium. Castle ('42) observed that during the first one or two hours of this stage helical growth reverses direction, being at first right-handed (the sporangium turning counterclockwise, as seen from above), and later left-handed. Accordingly, he subdivided stage IV into stages IVa and IVb.

The growth rate during stage IV has been described (Oort, '31) as reaching a steady value within a few hours after resumption of growth, this value being somewhat variable from specimen to specimen, and lying in the range from 2 to 4 mm per hour. Employing the two methods described in the preceding section, we have found that the growth rate increases quite uniformly from about 1 mm per hour to 4 mm per hour during a period of 10 hours, and then remains constant for some time. Unfortunately, by the time this plateau is reached, the specimens are too tall and mechanically unstable for the type of precision measurements to be reported. The specimens used in our experiments were somewhat younger, the speed increasing by about 25% during three to 4 hours, the maximum duration of one experiment.

Our data are not seriously affected by the fact that they are not taken under perfect steady state conditions, because this slow change in overall speed distorts our vertical scale

in a slow and uniform manner, and the shift does not affect the comparison of stretch and twist, since these are measured simultaneously at the same positions.

Length of the growing zone

Related to the question of the constancy of the growth rate is that of the constancy of the length of the growing zone. Buder ('46) has claimed that the ratio of the growth rate to the length of the GZ is constant from specimen to specimen, and within one specimen is constant as a function of time.

We have found (1) that the length of GZ varies appreciably within short intervals. Thus, by observing series of markers which follow each other at about half hour intervals, differences of 10% are observed; (2) during the gradual increase in growth rate mentioned in the preceding section the length of GZ does at first increase proportionally, but fails to do so in the later stages.

In sum, V/G varies between 0.015 and 0.027/min., with somewhat less variation within one specimen during its entire history, and with short term variations of about 10%.

Stretch and twist

Description of a typical experiment. Markers are placed on the stalk, one or two on its upper nongrowing part, to serve as reference marks, and one or two as near as possible to the upper end of the GZ (100 to 125 μ from the sporangium). The specimen is then centered on the turntable, illuminated bilaterally with an intensity $I = -3$, and the turntable put in motion at one turn per min. After at least two hours the markers are inspected periodically until it is noted that one of them has started to move. Thus, one usually misses the first 1% of the movement.

From the moment that a marker has begun its movement, its distance from the reference marker and its azimuth relative to this marker are measured continuously until it has reached the lower end of the GZ. The azimuth is determined by clock-

ing the transit times t_1 and t_2 of the reference marker and the moving marker. The difference $t_2 - t_1$ is the azimuth difference at time t_2, i.e., $\alpha(t_2)$.

We now wish to determine the vertical distance between the two markers at exactly this time t_2. The vertical position of the reference marker is measured with the ocular micrometer at the transits preceding and following that of the moving

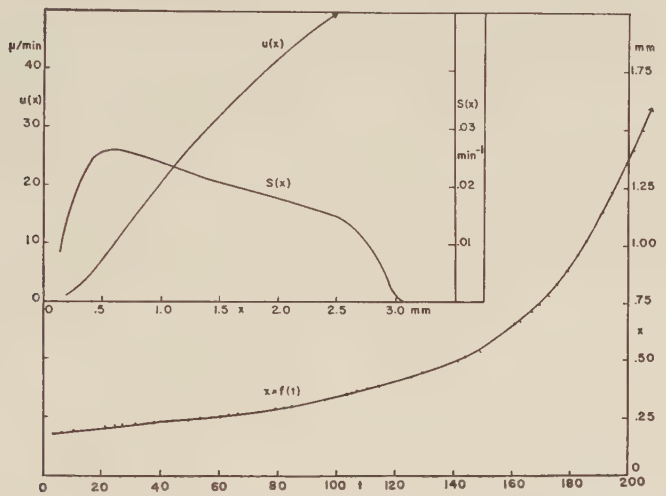

Fig. 3 The vertical coordinate of a marker, x(t), the speed distribution, u(x), and the stretch distribution, S(x), for experiment A; x(t) is measured directly; dx/dt plotted versus x, gives u(x); du/dx gixes S(x).

marker, and its position for the time of transit of the moving marker is inferred by interpolation. Similarly, the vertical position of the moving marker is determined at its moment of transit and the vertical coordinate x is the difference between these two quantities.

From these data $\alpha(t)$ and x(t) are obtained and stretch and twist as a function of position are evaluated by the procedures outlined in the preceding section.

Results. Figure 3 illustrates the determination of the stretch distribution by giving the functions x(t), u(x), and S(x) of experiment A. Figure 4 similarly illustrates the de-

termination of the twist distribution by giving the functions $\alpha(x)$, $w(x)$, and $T(x)$ for the same experiment. Figure 5 summarizes the results for 4 different specimens, giving only stretch and twist distributions.

The functions $S(x)$ and $T(x)$ each involve two numerical differentiations, and their determinations are therefore not very precise. The differences from specimen to specimen are, however, reliable. The results may be summarized as follows: the stretch functions start from a low value at the origin, go

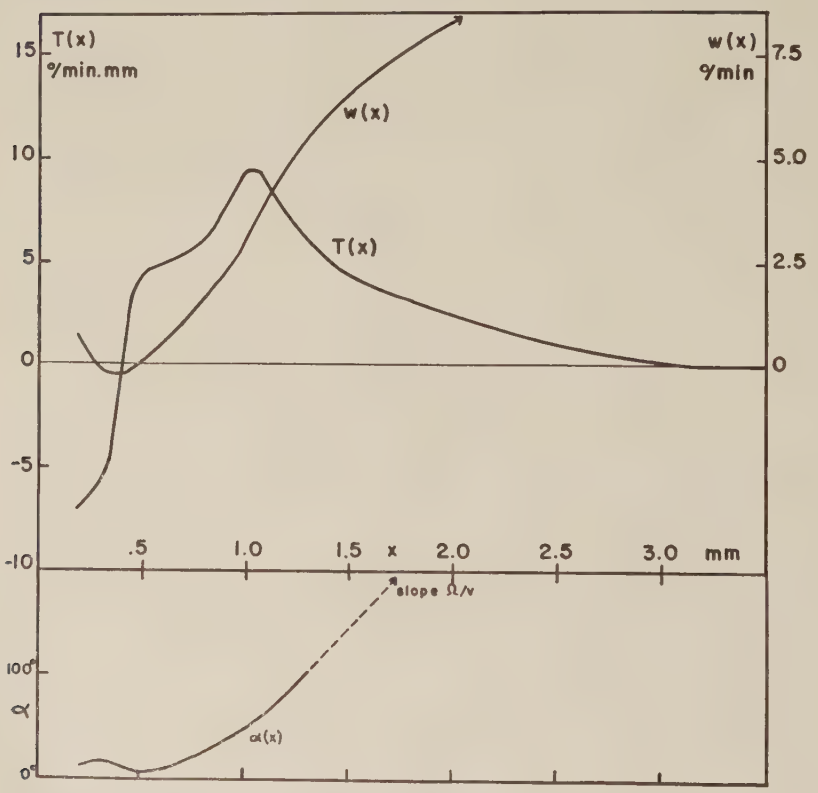

Fig. 4 The azimuth as a function of the vertical coordinate, α (x), the angular velocity as a function of the vertical coordinate, w(x), and the twist distribution, T(x), for experiment A. The function $\alpha(t)$ and x(t) are determined directly. $\alpha(t)$ is plotted in figure 6 (A1), x(t) in figure 3. $\alpha(x)$ is derived from these by inversion and substitution. $w(x) = d\alpha/dt$ plotted versus x. $T(x) = dw/dx$.

through a more or less pronounced maximum at approximately $x = 0.4$ mm and then decline very gradually to zero at the lower end of the GZ.

The twist function presumably also starts from small values and reaches *negative* values during the first few tenths of a millimeter. It then turns positive, reaching a broad maximum at a position somewhat lower than that of the stretch maximum, and declines to zero faster than the stretch.

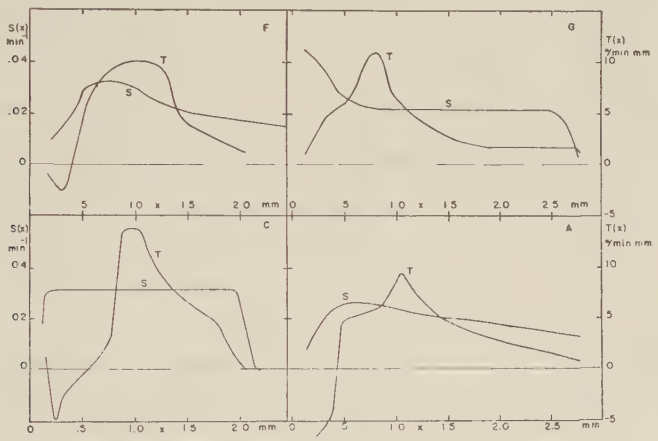

Fig. 5 Stretch and twist distributions in four specimens. Duration of experiments: between 150 and 250 minutes; growth speeds: between 3 and 4.5 mm per hour.

The curious negative values of the twist in the uppermost portion of the GZ are undoubtedly real. To demonstrate this convincingly, the functions $\alpha(t)$ for 7 experiments are presented in figure 6. During the early phases of the marker travel, α is at first constant and then decreases by very appreciable amounts in several experiments. The angular speed drops from zero to negative values and the twist is negative. It should be noted that the terminal angular velocity was positive in each of these cases. Indeed when the marker is near the bottom of the GZ, $\alpha(t)$ increases linearly with time, its slope being almost equal to the angular velocity of the sporangium. This final slope is similar in all of the experiments.

It seems likely that these negative values of the twist function are related to the transition from stage IVa to stage IVb. The transition presumably does not occur simultaneously for all parts of the GZ, and specifically the top part of the GZ seems to lag in this respect.

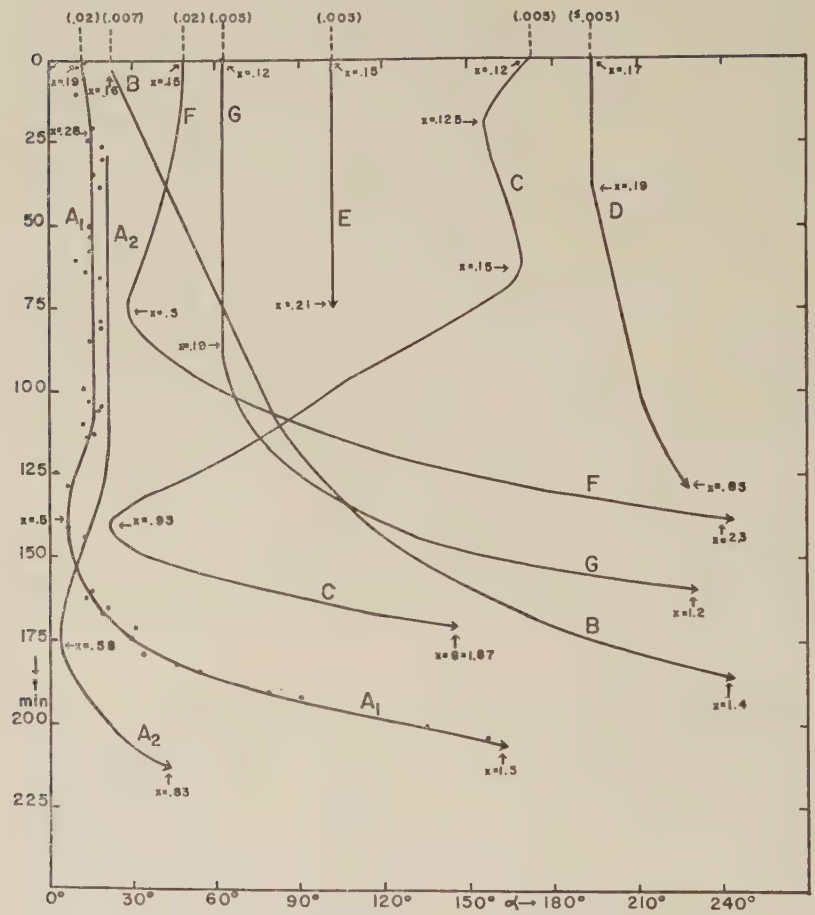

Fig. 6 The azimuth angle versus time, $\alpha(t)$, for seven specimens. Only the early parts are given, to illustrate the negative twist. For experiment A the experimental points are given to illustrate the experimental accuracy. In experiment A two markers (A1 and A2) were followed. The values in parentheses on top are the u/V values at the beginning of each experiment. Only in experiment C are the data plotted for the whole GZ: due to a strong negative twist the marker azimuth was smaller at the bottom of the GZ than when the marker started near the sporangium. Nevertheless the final angular velocity is similar to the other ones.

Distribution of the growth responses

We now turn from the steady state, and its distribution of stretch and twist, to the growth response. Here too, we have to distinguish between responses in stretch and responses in twist. The response in stretch was discovered by Blaauw ('14). That there exists a corresponding response in twist was demonstrated many years later by Oort ('31). In the introduction it was pointed out that these responses constitute the sum of the responses of material sections, and that these responses cannot be identical for all sections. Ideally we should mark a section by two markers at its upper and lower edges, and follow both these markers simultaneously. In practice it is easier to follow a single marker. Its response, measured relative to the ground, represents the *integral* of the responses of all sections *below* the marker. If these responses are plotted as a function of the position, the *distribution* of the response can be obtained by differentiation of the resulting curve with respect to position. These measurements can be done most efficiently by using periodic stimulations, rather than isolated stimuli. Under periodic stimulation the GZ as a whole exhibits a periodic response with characteristic shape, amplitude, and phase relative to the stimulus. If the period between stimulations is short, the marker does not move appreciably in the GZ during one period and its response curve can be referred to a particular position. During the first runs of this kind measurements were taken every half minute to ascertain the shape of the response curve at each level. Figure 7a shows the results of a run, during which the stretch response was measured, figure 7b a similar run for the twist response. In figure 7a the first curve gives the response curve at the sporangium. During each period the speed varies between 65% and 135% of the average speed. The minimum occurs at 2.75 min., and the maximum at 4.75 min. after the beginning of the stimulus. The curve is very nearly a sine curve. The other curves represent the response curves of the marker at lower levels.

The results show, on the whole, a remarkable similarity of the response curves throughout the whole GZ. Specifically, they show a constant phase relation between stimulus and response. Only at the lowest position do we see a systematic deviation. Here, in fact, the response is not strictly periodic. The speed at the end of the period is systematically lower than at the beginning. This has a simple explanation: in this region the marker distance from the sporangium increases appreciably during one period. The marker thus moves in one cycle to a region of lower speed and amplitude relative to ground.

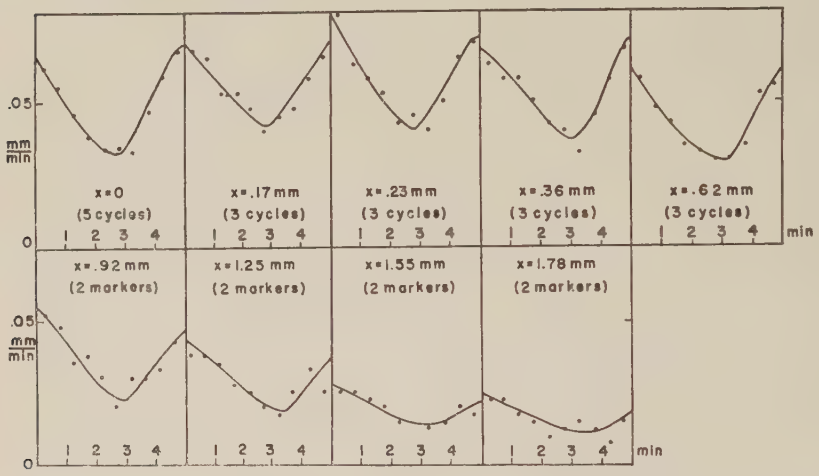

Fig. 7a Growth response curves for markers at various positions in the GZ. Periodic stimulations. Period 5 min. Bilateral conditioning illumination, I = — 5. Stimulus bilateral, I = 0, duration 15 sec. The first curve (response of the top), average over 5 periods. Next 4 curves, three successive periods used to obtain averages for each particular level. For the last 4 curves the marker speed was too great to do such averaging. Instead, a second marker was used on the same specimen after the first had completed its run and its responses were measured at positions corresponding to those of the first. These curves, therefore, are averages of only two periods, and of two different markers. Total duration 175 min. Growth speed 3.4 mm per hour. Since the specimens sometimes exhibit a spontaneous tropic oscillation with a period of 5 minutes (see footnote p. 369) it was thought that illuminations with the same period might produce singular results. Therefore the same type of experiment was performed with a 4 min. and with a 7 min. periodic stimulation, giving substantially the same results.

The amplitude of the marker speeds decreases as we pass from the top to the bottom of the GZ, but this decrease is not in proportion to the decrease in the average marker speed. This is particularly apparent for the two curves at x = 0.62 mm and 0.92 mm, where the average marker speed is appreciably lower than at the top, while the amplitude of the speed is practically the same as at the top. To study this more closely, numerous further runs were made, but in these the

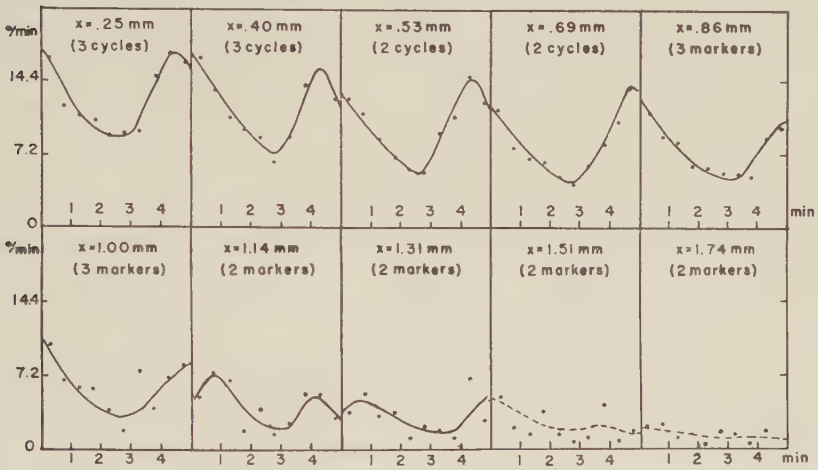

Fig. 7b Spiral growth response curves: measurements of the marker angular velocity under the same experimental conditions as in figure 7a. Total duration 95 minutes.

response curves were not followed in detail. Instead, only two measurements were taken during each cycle, at 1.25 and 3.75 min. after the beginning of stimulus. These times are chosen so as to bracket the periods of above average and below average growth rate, respectively. In some runs the top of the sporangium was followed throughout, taking measurements 5 secs. after those on the marker. In other runs, measurements on the top were only taken occasionally, to check on variations of the amplitude at the top. In each 5-min. interval there was taken also one measurement of x, usually at the beginning of the stimulus. The results of these measure-

ments for six experiments are given in figure 8. In the last two of these experiments the markers were followed down only to $x = 1$ mm. Both amplitude $A(x)$, and average of the marker speed $V(x)$ are plotted.

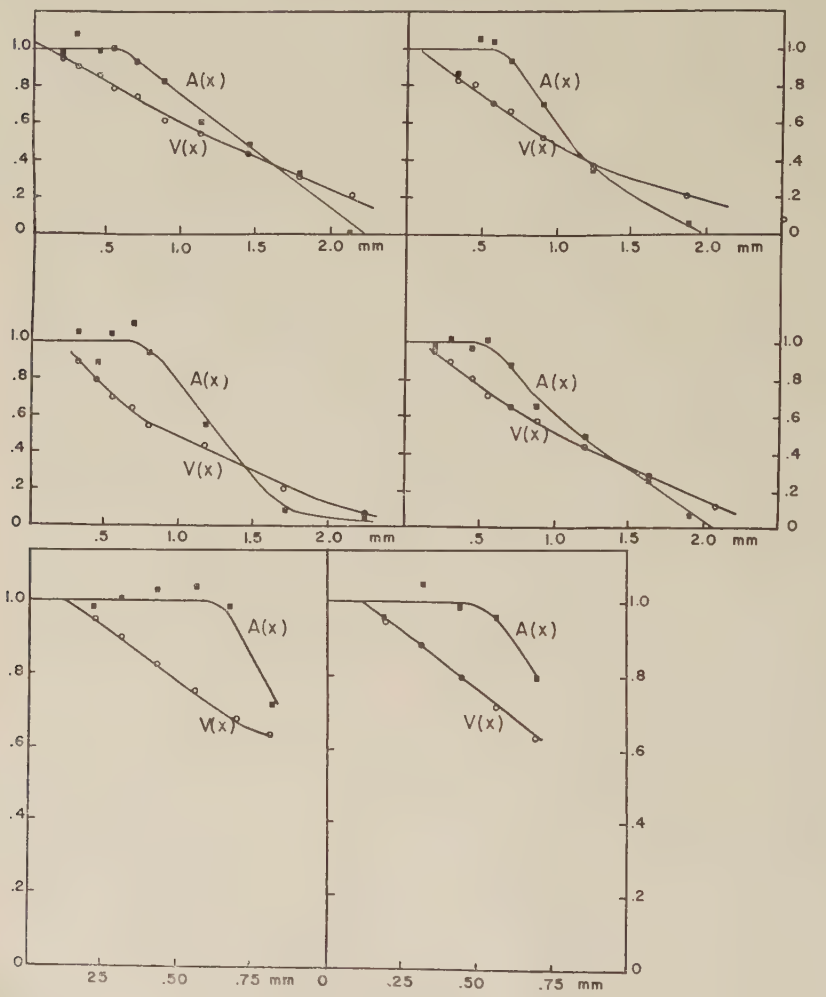

Fig. 8 Growth speeds, $V(x)$, and growth response amplitude, $A(x)$, as functions of position for markers on six specimens. Both V and A are normalized to unity at the sporangium. Each point in upper half of the GZ is the average of 5 to 7 consecutive periods. In the lower half of the GZ the number of periods averaged decreases, for the reasons given with figure 7. The absolute values of $V(x)$ and $A(x)$ are very similar to figure 7a values.

The average speeds are determined more accurately than the speed amplitude, as seen by the scatter of the points. This is inherent in the problem. Both determinations depend on measurements of position. An error in one such measurement increases the estimate of the speed in one period and decreases it in the next, and thus disappears when the average of two periods is formed. In contrast, when the amplitude of the speed *variation* is determined, an error in one position measurement changes the speed in the preceding and the succeeding interval in opposite directions, and these errors are *added* when the amplitude of speed variation is determined. Each single error in measurement of position is thus doubled in the determination of speed amplitude. Moreover, the error spreads to both neighboring intervals with the *same* sign, thus simulating a persistent trend in the speed amplitude.

In spite of these difficulties in the determination of the speed amplitude, the results presented in figure 8 are very clear. The speed amplitude is constant down to about 0.65 mm below the top and at this point the average speed has already decreased to about 65%. Moreover, the slope of V(x) versus x is steepest above this point, indicating that it is the region where the stretch function has its maximum. We conclude, therefore, that the growth response is absent from this region of maximum stretch.

Distribution of spiral growth response

Figure 7b shows the results of a preliminary experiment with measurements of the spiral growth response taken every half minute. The results are very similar to the results found for the growth response (fig. 7a). The only difference is that the maximum and the minimum seem to occur 15 seconds earlier in this case. The response starts also at about $x = 0.65$ mm and ends somewhere between $x = 1.5$ and 2.0 mm.

The distribution of the spiral growth response in the growing zone is very similar to the distribution of the growth response. To compare them directly the two responses have been measured simultaneously on the same specimen.

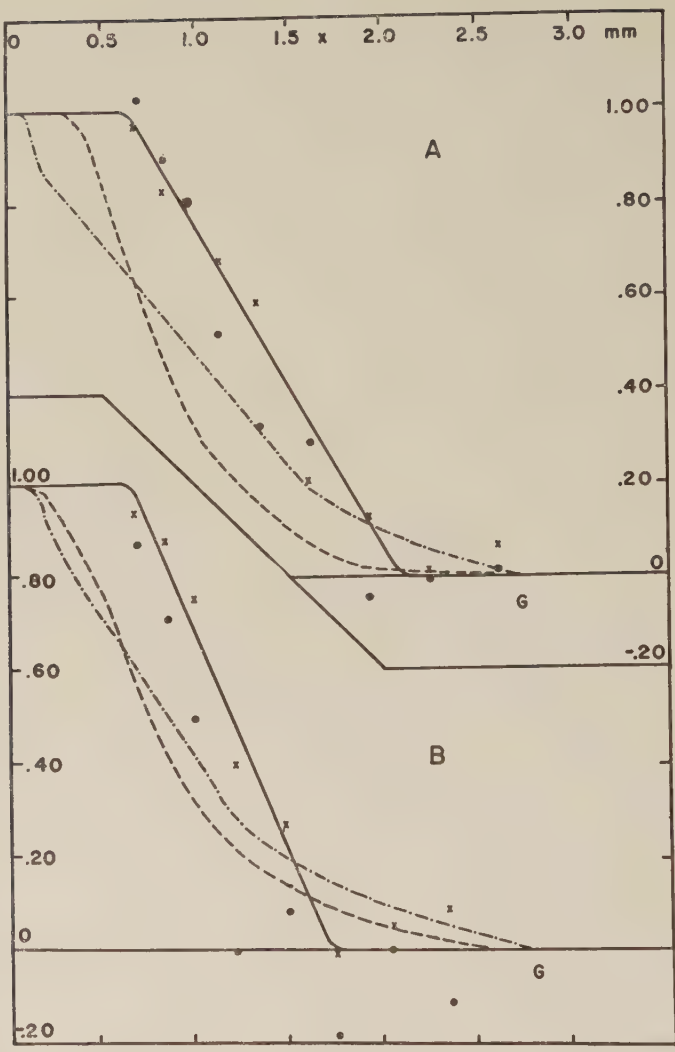

Fig. 9 Spiral growth response (·), growth response (x), angular velocity (– – –) and growth rate (– · – · –), as functions of the position x. The amplitudes have been normalized in the following way: the amplitudes were constant down to x = 0.7 mm in agreement with figures 7a and b. Their average in the uppermost 0.65 mm was taken as unity. Furthermore, in experiment A the two amplitudes were measured for a marker above the GZ immediately before and immediately after the experiment: the amplitudes thus found for this reference marker are almost (less than 5% difference) equal to the above averages. Two experiments. Duration 100 minutes each. Growth speed 4 mm per hour. The absolute values of these four functions are very similar to figure 7a and figure 7b values.

Using the same periodic illumination, the azimuth of the marker was measured at 1.00 and 3.50 min. after the beginning of the signal, while as previously, the vertical position was measured at 1.25 and 3.75 min. The azimuth readings were taken 15 seconds earlier than the position measurements in order to get the maximum possible spiral growth response amplitude.

In addition, once per cycle a reference marker was measured to obtain the average growth speed and angular velocity at the sporangium per 5 minutes interval. In this way a $V(x)$ curve, as used in the presentation of the growth response experiment, could be plotted, as well as an $\Omega(x)$ curve (defined in a similar way, as the ratio of the marker angular velocity to the simultaneous reference marker angular velocity).

Figure 9 shows the results of two such experiments.

All the features already found earlier for the stretch, the twist and the growth response can be recognized here. Furthermore, it seems that the amplitude of the spiral growth response decreases a little faster than that of the growth response.

SUMMARY AND DISCUSSION

Our results may be summarized somewhat schematically as follows.

1. Stretch occurs in the region between $x = 0.1$ and $x = 3$ mm, this region being by definition the GZ.

2. The intensity of stretch is maximal around 0.5 mm, remains on a plateau between 0.7 and 1.9 mm, and then declines to zero.

3. Twist is zero or negative where the stretch is maximal. Twist has a maximum around $x = 0.75$ mm in a region where the stretch, decreasing from its maximum, reaches the plateau. Twist then declines to zero faster than the stretch. In the lowest portion of the GZ twist is negligible compared to stretch.

4. The stretch response is confined to a region extending from 0.65 to 1.95 mm, i.e., to a region representing only 45%

of the total GZ. This region is almost the same as the stretch plateau, and the stretch response is nearly constant in this region.

5. The twist response is confined to an even narrower region, extending from 0.65 to 1.85 mm, and decreases faster than the growth response.

The steady state experiments show clearly that stretch and twist are only loosely connected phenomena. Stretch and twist are not proportional to each other, their maxima are located in different regions of the GZ and the twist is zero or negative where the stretch is maximal. The finding of Castle ('37) of a proportionality between stretch and twist is due to lesser accuracy inherent in the technique employed, particularly for the study of the uppermost region of the GZ, where the deviation from proportionality is most pronounced.

Of principal interest is the result that there is neither stretch nor twist response where stretch itself is maximal.

The response consists in changes in the intensity of stretch and twist in a region 1.3 mm long, starting at 0.65 mm from the sporangium. This region of the GZ constitutes the *growth response zone.* The *average* intensity of stretch in this zone is about 0.025/min. The observed variation of overall growth speed during a cycle is about 35 μ/min. This implies a variation of the stretch rate between the extreme values 0.040 and 0.010/min. (fig. 10).

This variation in stretch implies a small fluctuation in the length of the GZ. The boundary below which there is no stretch presumably advances at a constant speed, equal to the average growth speed V. During the half cycle in which the speed is above average, the sporangium advances 0.075 mm more than during the other half cycle. Thus the true length of this GZ varies by this amount (about 3% of its total length) during each cycle. This is too small to be verified directly. One may ask, though, whether this variation in length represents a true variation in *growth,* i.e., in the synthesis of new primary wall material. It is conceivable that this new synthesis goes on at a uniform rate and that the variations in

stretch rate result from variations in the elastic constants of the primary wall, or even in the turgor pressure.

Roelefson ('49–'50) has studied the elastic properties of the intact sporangiophore in an ingenious manner. It is known that the sporangiophores can be severed from the mycelium without losing their turgor. Roelefson inserted the lower portion of the sporangiophore in an "iron lung" and studied stretch and twist of the GZ when the turgor was increased by several atmospheres pressure applied below. His results suggest that the relation between stretch and twist, resulting from

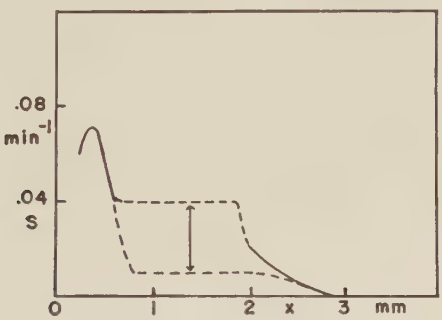

Fig. 10 Schematic representation of the changes in the distribution of stretch in response to a periodic illumination program. The stretch distribution oscillates between the upper and the lower dotted curves. (The values of the stretch in the upper part of the sporangiophore are taken from figure 8. They are higher than those obtained in the steady state experiments (fig. 5). The reason for this difference is unknown.)

variations in *turgor,* is different from their relation during *growth.* A variation in turgor causes much less twist per unit of stretch than occurs during growth.

In the experiments of Roelefson the bottom parts of the sporangiophores were in air and the sporangiophores were not growing. Gruen ('57) has recently reported that sporangiophores severed from the mycelium and dipped into distilled water will grow at normal rate for about 24 hours. We have verified this and found, moreover, that the sporangiophores remain perfectly phototropic during this time. It would therefore be possible to do the "iron lung" experiment of Roelefson

with growing sporangiophores responding to light, and to compare very directly stretch and twist responses to light with those to turgor variations, and further, to measure whether the variations in growth rate, due to light, are accompanied by variations in the elastic response. Such a study should be very helpful in nailing down the nature of the process which is directly controlled by light.

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PATHWAYS OF TERMINAL RESPIRATION IN MARINE INVERTEBRATES

I. THE RESPIRATORY SYSTEM IN CEPHALOPODS [1]

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TEN FIGURES

The blood respiratory pigments in living organisms are essentially iron and copper proteins. With the exception of hemerythrin, the iron pigments are hemoproteins of different kinds: hemoglobin in all classes of Vertebrates, herythrocruorin in a number of Invertebrates and chlorocruorin in some worms (Chetopods). The copper pigments, the hemocyanins, are found in several groups of Invertebrates, sometimes very closely related to those groups containing iron pigment in the blood. No chemical relationship exists between hemoproteins and hemocyanins since in hemocyanin no porphyrin is present and the copper is directly linked to the protein (Nicolaus, '57). The distribution of these two blood pigments is not consistent with the zoological classification of living forms.

The respiratory enzymes which can transfer electrons to molecular oxygen in terminal respiration are essentially iron porphyrin compounds and copper oxidases. These two groups of proteins, so different from each other but having the same function, are chemically related to hemoproteins and hemocyanins respectively (Warburg, '46).

It is generally accepted that hemoproteins have been formed from iron porphyrin respiratory enzymes (Keilin, '25). The

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Dedicated to E. S. Guzman Barron.

same can be assumed for hemocyanin as derived from copper oxidases. This suggests that in those organisms which have a copper protein as oxygen carrier in the blood, the cells might also contain enzymes which are capable of transporting electrons in terminal respiration by means of copper (Warburg, '46).

In the present work we have investigated the pathway of electron transfer in the last steps of terminal respiration of Cephalopods. It includes: quantitative determination of copper and iron in the organs of the animal; distribution of the flavins as riboflavin, FMN and FAD; effect of cyanide and of carbon monoxide on the respiration of tissue slices; effect of light on carbon monoxide inhibition; preparation of isolated particles from the organs; spectrophotometric detection of the cytochrome system present in them; measurement of succinic dehydrogenase and cytochrome oxidase activities; detection of polyphenol oxidase activity of the organs and of hemocyanin; detection and measurement of a DPN dependent quinone reductase and its possible coupling with polyphenol oxidase.

MATERIAL

In most of the experiments the animal used was *Octopus vulgaris;* in a few cases *Eledone moschata* and *Octopus macropus* (fig. 1).

The blood was drained off by introducing a canula into the dorsal aorta of the animal kept under water. After bleeding another canula was introduced into the vessel and filtered sea water was allowed to flow in the cephalic direction under a pressure of 50 cm water until the effluent fluid did not contain any hemocyanin. The perfusion took generally half an hour and 1–2 liters of sea water were used.

1. *Preparation of isolated particles*

(a) Body muscle. Because of the peculiar properties of the proteins, the classical method of differential centrifugation in a sucrose medium could not be used. Homogenization

Fig. 1 A preparation of *Octopus vulgaris* opened from the ventral side to show the internal organs. The tentacles have been removed. (From the Museum of the Zoological Station). 1, Eye with the optical lobe; 2, salivary gland; 3, gill; 4, kidney; 5, branchial heart; 6, the place from which hepatopancreas has been removed.

of the muscle in 0.7 M sucrose gave indeed an extremely viscous and swollen paste which refused any centrifugation. Phosphate buffer gave better results. By low speed centrifugation the large undispersed pieces of muscle could be separated from the fine granular material and pieces of fibrils, which can be collected by higher speed centrifugation. After several attempts the most suitable procedure was found to be as follows.

The muscle of the mantle and tentacles was carefully skinned and frozen at —20°C, then ground in a meat grinder. Freezing was found to be necessary since the tissue was very slimy and resisted grinding. All further manipulations were performed at 3°C. The ground material was weighed and suspended in 5 volumes of cold distilled water for 30 min. The suspension was then passed through cheese cloth and the solid material resuspended in the same volume of water. This treatment was repeated 5 times. At the end of this procedure, the water was squeezed through muslin and the material was homogenized in cold 0.1 M phosphate buffer pH 7.3 in a Waring blendor for 7 min. About 2.5 l of buffer were used per kg of frozen muscle. The thick suspension so obtained was centrifuged at 2000 × g for 30 min; the sediment was collected, resuspended in an equal volume of buffer and again centrifuged. The combined supernatants were centrifuged at 30,000 × g for 1 hr. in the Spinco preparative centrifuge. The small sediment was washed in phosphate buffer and then suspended in an amount of the same buffer to give approximately 40–50 mg dry wt. per ml. This preparation can be stored at —20°C for several days.

(b) Central heart. The ventricles from 36 animals were used. Homogenization was performed in a porcelain mortar with washed sand and 5 volumes of 0.1 M phosphate buffer pH 7.3. Sucrose 0.6 M can be also used. After low speed centrifugation the supernatant was centrifuged at 30,000 × g for 1 hr.; the small sediment was washed, centrifuged again and suspended in 5 ml phosphate buffer.

(c) Hepatopancreas. The organ was homogenized in 5 volumes of 0.7 M sucrose using a glass homogenizer with a teflon pestle. After centrifugation at 2000 × g for 20 min., the suspension separated into three layers: a bottom layer contained large unbroken particles, the upper layer contained fats and the middle one was formed by a clear suspension of fine particles. These were collected by centrifugation at 30,000 × g for 1 hr., then washed twice with sucrose and finally resuspended in a small volume of phosphate buffer.

The suspension was found to be still not homogeneous. Further differential centrifugation in a density gradient of sucrose according to the method described by Kuff and Schneider ('54) gave 5 distinctly separated layers. One-milliliter portions of the original suspension were layered over the contents of cellophane tubes having from bottom to top 1 ml each of 2.25, 2.0, 1.75 and 1.5 M sucrose and centrifuged for 1 hr. in the Spinco SW 39 rotor. The tubes were cut using a modification of the cutter described by Randolph and Ryan ('50) and the layers collected for further determinations.

(d) Other organs. All attempts to prepare extracts from the salivary glands were without success. Homogenizations of the organs in a Waring blendor or in a mortar with water or saline or sucrose gave an extremely viscous material which swelled continously after further addition of liquid. Attempts to separate particles from this thick suspension by centrifugation in a density gradient were also unsuccessful.

The isolation of particles from gills and kidneys was also very difficult owing to the great amount of mucus present in these organs. Even after washing with large quantities of water or saline, the mucus still made any separation of the particles by differential centrifugation impossible.

2. Preparation of acetone powders

The ordinary method used for preparation of acetone powders from mammalian organs was applied with success to the hepatopancreas. The organ was homogenized in a Waring

blendor with 10 vol. of cold (—20°C) acetone, filtered, washed with another 5 vol. of cold acetone and dried in a desiccator under vacuum. With this procedure the fats and pigments were removed and the pale yellow powder retained several enzyme activities (amino-oxidases, amino-acid oxidases, etc.).

Attempts to prepare acetone powders from salivary glands were made. When extracted, a thick and swollen solution was obtained as during the preparation of crude homogenates.

3. Preparation of slices

Using a thin razor blade and cutting by hand, slices of a thickness of about 0.5 mm were made from the salivary glands, the central heart and the body muscle. They were collected in chilled sea water or mammalian Ringer solution which were kept thoroughly oxygenated by bubbling oxygen until used for manometric experiments.

The hepatopancreas as well as the branchial hearts are so soft as to be almost fluid and slices cannot be prepared from them. The gills and the kidneys are also not suitable for tissue slices preparations.

4. Purification of hemocyanin from the blood

Lymphocytes were removed from hemolymph by centrifugation. The supernatant was dialysed against running tap water for 12 hrs. and the small white precipitate which formed was discarded. Pure hemocyanin was prepared either by precipitation with ammonium sulphate and crystallization according to Kubowitz ('38) or by long-term high speed centrifugation of the clear solution (4–5 hr. in the Spinco preparative centrifuge).

METHODS

Copper was determined according to Eden and Green ('40); iron with a modification of Lorber's method ('27) using sulphosalicylic acid as reagent. Nitrogen was estimated by titration after distillation in a micro-Kjeldahl apparatus; proteins were determined spectrophotometrically with the

formula given by Kalckar ('47). Flavins were determined fluorometrically according to the method described by Bessey et al. ('49).

Oxygen uptake determination. The oxygen uptake was measured by the conventional Warburg method in presence of alkali in the central well and air or oxygen as gas phase. All measurements were made at 24°C. The slices were suspended in 3 ml of filtered sea water; particles were suspended in a convenient buffer.

Sensitivity to cyanide was studied in a gas phase of oxygen and the inhibitor was added to the sea water in the main compartment of the vessel. In every case the pH of cyanide was adjusted to pH 7.8 and the central well contained a mixture of alkali and cyanide according to the instructions given by Krebs ('35).

The effect of carbon monoxide on tissue respiration and its reversibility by light was studied according to Warburg ('26). A mixture of carbon monoxide and oxygen (95% : 5%) was prepared and the mixture gassed in the vessels attached to the manometers. Blank vessels containing only the gas mixture and KOH in the central well were made for each experiment. The amount of gas taken up was substracted from the oxygen uptake of the tissues. Control experiments were run in a mixture of nitrogen and oxygen (95% : 5%).

For the study of photoreversibility of carbon monoxide inhibition, the light source was a Philips neon lamp fitted for underwater use, placed in the water bath directly below the vessel. Monochromatic light (435 mμ) was obtained with interposed filters. The effect of the dark was studied either by turning off the lamp at intervals or with control vessels made lightproof with carbon black varnish.

Difference spectra. Because of the great light scattering effect of the particle suspensions used, absolute spectra of the cytochromes cannot be taken. However, as shown by Chance ('54), the light scattering by a turbid suspension does not interfere with measurements of the change in optical density as the pigments go from the oxidized to the reduced state

if the light scattering is the same in the two forms. We have been able to take the difference spectra of the cytochromes in our particle suspensions by measuring the difference of optical density (from 380 to 640 mμ) between a suspension containing the pigments in the reduced state and another suspension having the same light scattering properties but in which the pigments were in the oxidized state. All spectrophotometric measurements were made with a DU Beckman spectrophotometer with a photomultiplier attachment using 1 cm light path cuvettes especially designed for work in anaerobiosis. The optical density increment plotted against the wavelengths gave difference spectra of the pigments present.

The pigments were reduced with sodium hydrosulfite or succinate, ascorbic acid and reduced DPN. The cuvette was previously evacuated and filled with hydrogen. The difference spectra of the carbon monoxide compound of cytochrome oxidase were taken after reduction with ascorbic acid and saturation with carbon monoxide.

Enzyme activity determinations. The activity of the complete succinic oxidase system and of succinic dehydrogenase were measured manometrically in the presence of excess cytochrome *c* and of KCN and MB respectively (Slater, '49a). The activity of cytochrome oxidase was determined with ascorbic acid (Slater, '49b), hydroquinone or p-phenylen-diamine as substrates.

Quinone reductase activity was measured spectrophotometrically according to the method described by Wosilait and Nason ('54). Extracts were prepared from the tissues by centrifuging the Waring blendor homogenates at 3000 × g for 20 min. at 4°C. The reaction was started by the addition in a Beckman cuvette of 0.45 μM of p-quinone to a mixture containing 2.7 ml of 0.1 M phosphate buffer pH 6.5 plus 0.3 μM reduced DPN and tissue extracts to give a final volume of 3 ml. After addition of substrate, the decrease of O.D. at 340 mμ was measured at 30-sec intervals for the first 3 min. The formation of hydroquinone was also followed by observing the increase in O.D. at 290 mμ. The nonenzymatic rate was

determined as above except that the enzyme was omitted from the reaction mixture.

According to the definition of Wosilait and Nason, one unit of quinone-reductase is the amount of enzyme which results in a change of O.D. by 0.001 per minute calculated from the change between 15 and 45 seconds reading, and corrected for the non enzymatic rate. The specific activity is expressed as units per mg of protein as measured spectrophotometrically at 280 mμ.

Chemicals. DPN 60% purity was purchased from Pabst; DPNH was obtained by reduction of DPN with alcohol dehydrogenase and was isolated as the Tris salt according to Loewus et al. ('53); alcohol dehydrogenase was prepared from yeast after the method of Racker ('50); cytochrome *c* was prepared from beef heart according to Keilin and Hartree ('37) and its concentration estimated spectrophotometrically. Eastman Kodak sodium succinate was crystallized by alcohol precipitation. The riboflavin used as a standard in flavin determination was a commercial product; carbon monoxide was prepared by dropping formic acid into hot sulfuric acid and then washed by passing it through 10% sodium hydroxide.

RESULTS

Iron and copper content of the organs

Table 1 shows the iron and copper content of several organs of *Octopus vulgaris.* Hepatopancreas has the highest content of iron while no detectable traces of iron were found in the hemolymph. Branchial hearts also gave a high iron value; these organs have a deep red violet color due to a pigment containing most of the iron present in the organ which has been shown not to have a respiratory function. (A. Ghiretti-Magaldi, unpublished).

The kidney of Cephalopod molluscs form the environment for a puzzling phylum of minute multicellular organisms: the Dicyemid Mesozoa (Nouvel, '32). Octopus kidneys were almost full of Dicyemids. All attempts to free the organs

from these parasites (by washing, perfusion with sea water, centrifugation) were unsuccessful. Our results of chemical determinations made with this tissue, as well as those of the enzymatic activities must be accepted with this reservation. According to Emanuel and Martin ('56), Dicyemids contain copper, not iron.

The hepatopancreas is also the richest organ in copper. Its content is as high as that found for hemolymph. Hemolymph does not contain free copper; its copper content is that of hemocyanin.

TABLE 1

Iron and copper content of the organs and the hemolymph of Octopus
(γ per gm dry weight tissue)

ORGANS	FE	CU
Hepatopancreas	1920	2550
Branchial heart	399	93
Gill	188	111
Central heart	160	43
Kidney	112	48
Body muscle	47	28
Hemolymph	0	2450

Flavins

Table 2 shows the total flavin content (riboflavin + FMN + FAD) and the free riboflavin + FMN content of several organs as calculated in γ/gm wet tissue and in γ/gm dry tissue. The FAD content was calculated by difference and reported also as per cent of the total flavins. All figures are the average of at least 4 experimental values.

The richest organs in total flavins were the kidneys and hepatopancreas. The highest relative concentrations of FAD were found in the nervous system (brain and optic lobes) and in the central heart.

Endogenous respiration

1. Oxygen uptake. The respiration of *Octopus* tissues was found to be very sensitive to changes in oxygen tension (table

TABLE 2

FAD and non-FAD riboflavin fractions of several tissues of Octopus vulgaris

ORGANS	γ/GM WET TISSUE		γ/GM DRY TISSUE			FAD PER CENT OF THE TOTAL
	Total Riboflavin	FMN + free Riboflavin	Total Riboflavin	FMN + free Riboflavin	FAD	
Kidney	23.7 ± 5.2	12.1	134.1 ± 21.5	68.7	65.4	48
Hepatopancreas	23.9 ± 1.5	13.9	76.7 ± 1.8	48.4	28.3	37
Central heart	5.6 ± 0.4	1.7	32.7 ± 0.6	11.3	21.4	65
Gill	4.0 ± 0.2	1.7	21.8 ± 1.2	10.1	11.7	53
Branchial heart	3.7 ± 0.7	1.8	17.9 ± 2.6	8.6	9.3	52
Salivary gland	4.5 ± 0.6	1.8	15.8 ± 2.0	6.7	9.1	58
Optical ganglion	2.7 ± 0.4	0.9	14.7 ± 1.0	5.1	9.6	65
Brain	2.6 ± 0.1	0.9	14.6 ± 0.5	5.0	9.6	66
Body muscle	1.3 ± 0.3	0.5	6.7 ± 0.9	2.5	4.2	62

3). Most of the organs studied doubled their respiration when the gas phase of the Warburg vessels was changed from air to oxygen. The highest Q_{O_2} in oxygen were found for the optic ganglia, the kidneys, the salivary glands and the gills; they are of the same range of those found by Barron et al. ('53) for the heart of the squid. The respiration of branchial hearts does not increase in an oxygen phase; the body muscle has the lowest value, much lower than that of the muscle of Insects (Barron and Tahmisian, '48) but comparable with that of Tunicates (Fish et al., '51).

TABLE 3

The effect of oxygen tension on the respiration of tissues of Octopus vulgaris

ORGANS	Q_{O_2} (μl O_2/MG DR. WT./HR)	
	Air	Oxygen
Optical ganglion	1.86	4.76
Kidney	2.07	3.05
Salivary gland	0.83	2.81
Gill	1.64	2.43
Branchial heart	1.78	1.74
Branchial gland	1.13	1.42
Hepatopancreas	0.67	1.02
Mantle muscle	0.42	0.88
Central heart	1.57	
Eggs	0.51	

2. Inhibition by cyanide. Sensitivity to cyanide was found to be very high for almost all the organs studied where it is apparent that millimolar KCN inhibits respiration from 100% to 80% (table 4). The only exception is represented by the hepatopancreas where the inhibition of oxygen uptake is only 52% and is not increased by further tenfold increase in cyanide concentration.

3. Inhibition by CO and its light reversibility. Table 5 shows the results of some representative experiments with salivary gland slices. Salivary gland was found the most suitable organ for this kind of experiments. In 4 experiments 95% CO in the dark affected inhibition of respiration over a range

of 19–49%. In all cases this inhibition was completely or near completely reversed by light.

Properties of the isolated particles

1. Vital staining. The muscle particle preparations, observed under a Zeiss phase contrast microscope, appeared to consist of an homogeneous suspension of spherical particles and are essentially free of debris. Due to their small size it was not possible to observe individual staining of the particles. One milliliter of the particle suspension in phosphate buffer or sucrose was incubated at room temperature with Janus green B in small test tubes. The final concentration of the dye was 1: 20.000. If the tubes were centrifuged at once,

TABLE 4

The effect of KCN $1 \times 10^{-3} M$ on the oxygen consumption of the tissues of Octopus vulgaris. Gas phase: oxygen

ORGANS	Q_{O_2}	$\frac{CN}{Q_{O_2}}$	INHIB.
			%
Optical ganglion	4.60	0	100
Mantle muscle	0.98	0	100
Branchial gland	1.35	0.13	90
Kidney	3.01	0.33	89
Gill	2.35	0.26	88
Branchial heart	1.70	0.28	84
Salivary gland	2.74	0.58	80
Hepatopancreas	1.00	0.48	52

TABLE 5

The effect of CO: O_2 (95%: 5%) in dark and in light on the respiration of Octopus salivary glands slices. Q_{O_2} (μl O_2/mg dr.wt./hr.)

N. EXPERIMENT	$\frac{N_2: O_2}{95\%: 5\%}$ Q_{O_2}	CO DARK		CO LIGHT	
		Q_{O_2}	% inhib.	Q_{O_2}	% inhib.
O. macropus 1	0.88	0.54	39	0.83	5
O. macropus 2	0.99	0.50	49	0.89	10
O. vulgaris 1	0.98	0.80	19	1.06	0
O. vulgaris 2	0.94	0.60	36	0.92	0

the sediment stained blue and the supernatant was colorless. When the tubes were allowed to stand at room temperature for half an hour, the suspension became red and after centrifugation the sediment appeared red and the supernatant colorless. If oxygen was bubbled into the tubes, the dye remained blue. These observations are consistent with the behavior of mitochondria as reported by Cooperstein et al. ('53) for mammal preparations.

TABLE 6

Peaks in difference spectra of Octopus cytochromes. Wavelengths in mμ

REDUCING AGENT	VISIBLE			SORET		
	a		*β*	*γ*		
Succinate	605	560	423	445		
Dithionite	605	560	423	445	435	
DPNH	605	560	423	445		
Ascorbic acid	605	560	423	445		415

2. Difference spectra. The difference spectra of cytochromes in a suspension of particles from muscle in different experimental conditions are reported in figures 2–5 and table 6. In figure 2 reduction was obtained by addition in the absence of air (gas phase hydrogen) of solid sodium succinate or sodium hydrosulfite. In the visible region of the spectrum the peaks are observed at 605, 560 and 522–525 mμ. They correspond respectively to the α bands of cytochromes *a, b* and to the β bands of cytochromes *a, b* and *c*. In the Soret region only one band is present at 445 mμ which corresponds to the γ band of cytochrome a_3. A small shoulder at 485 mμ is very similar to the band attributed by Keilin and Hartree ('39) to an oxidized flavoprotein. It disappears on further reduction with sodium hydrosulfite. The presence of flavoproteins is demonstrated by the trough at about 470 mμ.

By further reduction with hydrosulfite an increase of the optical density of the peaks was observed, probably due to partial inactivation of the cytochromes present or to the

presence of particles devoid of succinic dehydrogenase and therefore not reducible by succinate. Moreover a shoulder appears at 435 mμ corresponding approximately to the γ band of cytochrome *b*.

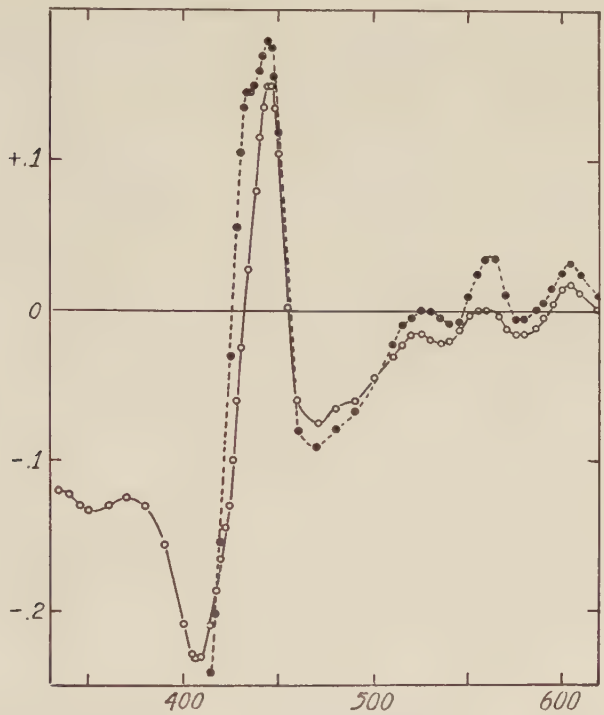

Fig. 2 Difference spectra of the cytochromes in a suspension of particles from body muscle. Solid line: reduction with sodium succinate. Dotted line: reduction with sodium hydrosulfite. Abscissa: wavelength in mμ; Ordinate: optical density.

Figure 3 shows the effect of reduction by addition of DPNH. The α bands of cytochromes *a*, *b* and *c* are observed as well as a small β band of *b* and *c*. The shoulder at 485 mμ and the sharp peak in the Soret region at 445 mμ are also present. The γ band of cytochrome *c* is not visible.

The spectrum obtained by reduction with ascorbic acid is reported in figure 4. In addition to the bands already observed after reduction with succinate and DPNH, it shows a

peak at 415 mμ corresponding to the γ band of cytochrome *c* and a large one at 345 mμ probably due to the presence of reduced pyridine nucleotides.

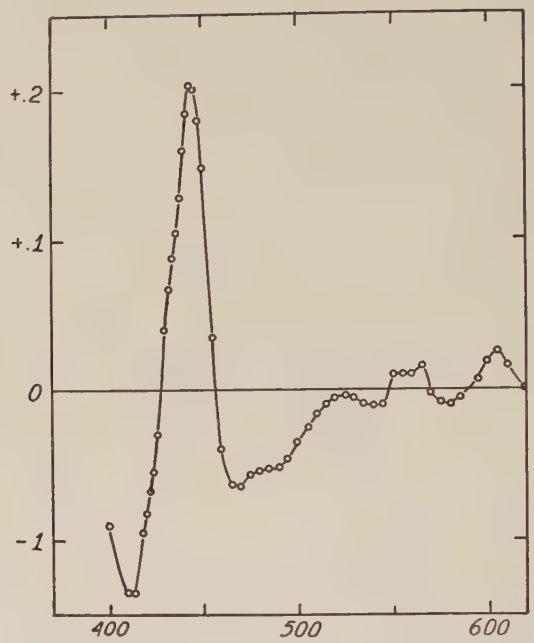

Fig. 3 Difference spectrum of the cytochromes in a suspension of particles from body muscle. Reduction with DPNH. Abscissa: wavelength in mμ; Ordinate: optical density.

Cytochrome a_3 was identified also by the spectral study of its CO compound. The particle suspension was treated with ascorbic acid and its spectrum was read against the oxidized form. Later on this was also reduced with ascorbic acid and then saturated with CO and the ascorbic acid plus CO compound was read against the first ascorbic acid treated suspension. A trough at 445 mμ and a peak at 430 mμ were found, both characteristic of the CO compound of cytochrome oxidase (fig. 5).

3. Enzymatic activities. (a) The oxidation of succinate. Sodium succinate was readily oxidized by the isolated particle preparation of several organs of Octopus. The activity

of the complete succinic oxidase system was measured in air with and without the addition of cytochrome *c*. Addition of cytochrome *c* increased the oxidation of succinate (fig. 6 and table 7). The optimum substrate concentration was found to be around 0.1 M; higher concentrations, up to 0.25 M, caused

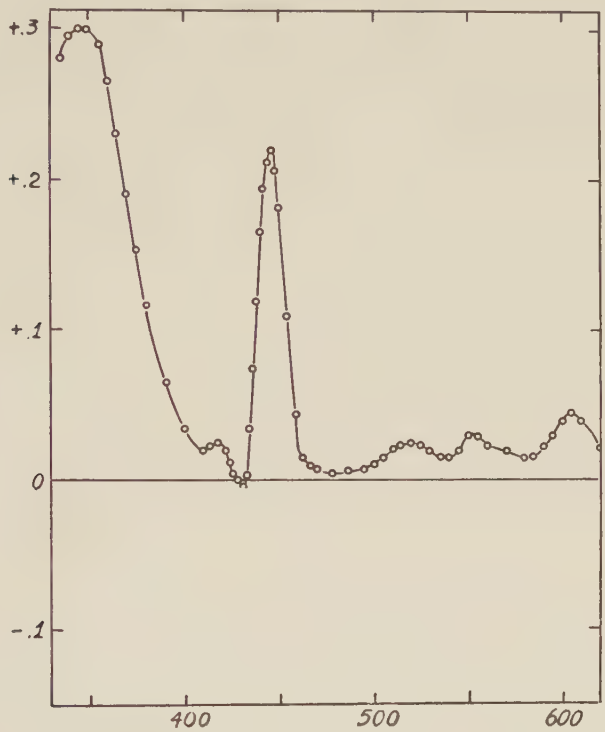

Fig. 4 Difference spectrum of the cytochromes in a suspension of particles from body muscle. Reduction with ascorbic acid. Abscissa: wavelength in mμ; Ordinate: optical density.

a definite inhibition. The action of pH was studied both for the complete succinic oxidase system and the succinic dehydrogenase activity, and the optimum was found at pH 8.2.

Crude homogenates, as well as particle preparations from hepatopancreas did not oxidize succinate either in absence or in the presence of cytochrome *c*. As mentioned before, the

particle suspension prepared from this organ is not homogeneous and by differential centrifugation in a density gradient solution of sucrose it gives 5 distinctly separated layers. The activity of each of them towards succinic acid was investigated spectrophotometrically according to Cooperstein

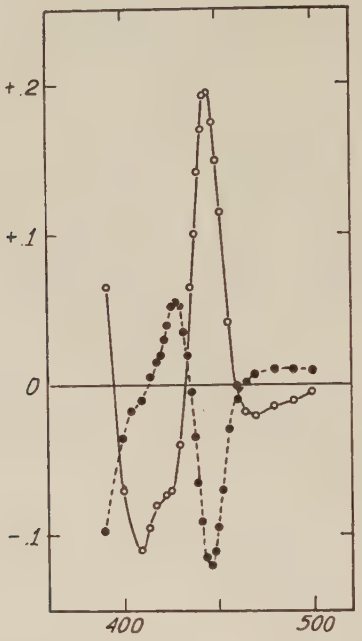

Fig. 5 Difference spectra of the cytochromes in a suspension of particles from body muscle treated with carbon monoxide. Solid line: ascorbic acid treated minus oxidized; dotted line: ascorbic acid + carbon monoxide treated minus ascorbic acid. Abscissa: wavelength in mμ; Ordinate: optical density.

et al. ('50). Only the fraction which sedimented in the bottom layer in 2.25 M sucrose has succinic dehydrogenase activity as demonstrated by the increase in O.D. at 550 mμ of added cytochrome *c*.

(b) Cytochrome oxidase activity. During the preparation of particles the concentration of cytochrome *c* is reduced to such a level that the cytochrome system does not function. The manometric assay of these particle suspensions for cyto-

chrome oxidase depends entirely on the fact that the addition of excess of cytochrome *c* causes an increase in the oxidation of substrates such as ascorbic acid, hydroquinone or p-phenylendiamine. This increment is taken as a measure of the cytochrome oxidase activity of the preparation.

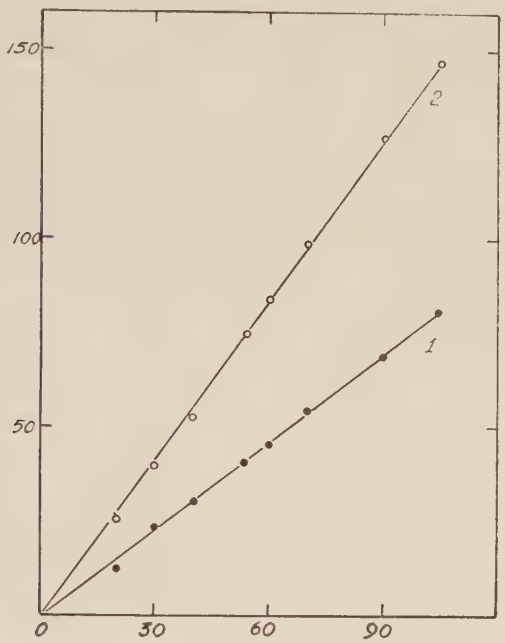

Fig. 6 The oxidation of succinic acid in absence (curve 1) and in presence of added cytochrome *c* (curve 2) by a particle preparation from body muscle. Final concentrations: phosphate buffer or tris buffer 0.15 M; succinate 0.05 M; Cyt. *c* $2.5 \times 10^{-5} = M$. Abscissa: time in min.; ordinate: oxygen uptake in μl.

Preliminary experiments were made to determine the quantity of oxidase preparation and the optimal phosphate buffer concentration required to give a convenient rate of oxygen uptake. Other experiments were made to determine the concentration of cytochrome *c* necessary to reach the saturation of cytochrome oxidase.

The cytochrome oxidase activity of the particles prepared from several organs was studied at pH 7.3 using the above

mentioned substrates. Figure 7 and table 8 show the results of typical experiments. Addition of cytochrome *c* causes a variable but definite increase in the oxidation of the substrates used.

TABLE 7

The effect of added mammal cytochrome c on the oxidation of succinate by particle suspensions of several organs of Octopus

Microliters of oxygen uptake in 1 hr. Final concentrations in the Warburg vessels: 0.15 M phosphate or tris buffer pH 7.3; 0.05 M succinate; 2.5×10^{-5} M cytochrome *c*. 0.5 ml of the original particle suspension were used. Gas phase: air. T = 25°C.

ORGANS	NO CYT. *c*	CYT. *c* ADDED
Body muscle 1	78.6	169.5
Body muscle 2	41.6	85.0
Central heart 1	26.5	172.0
Central heart 2	11.0	123.0
Kidney 1	21.8	34.6
Kidney 2	19.8	23.8
Branchial heart	37.0	36.8
Gill	2.1	10.0
Hepatopancreas	0	0

Since the possibility exists that the action of cytochrome *c* might be related to some indirect oxidation not related with cytochrome oxidase activity, a photoreversible inhibition by mixtures of carbon monoxide and oxygen was investigated. In figure 8 a typical experiment using a muscle particle suspension is reported. It shows that the CO inhibition is totally reversed by light. (See also table 9.)

Phenolase and quinone-reductase activities

1. Phenolase activity of the organs. The presence of phenolase in Cephalopods and its role in the formation of melanin has been established for many years. In 1933 Califano extracted from the ink gland of *Sepia* an enzyme which oxidized tyrosine at pH 6.9 and found that the process of melanin formation was similar to that described by Raper for plant tyrosinase. The kinetics of the reaction were also studied by Califano and Kertesz ('39). It was found that whereas ortho-

dihydric phenols were readily oxidized, the oxidation of monohydric phenols occurs only after a long induction period and that this period could be reduced by adding catalytic amounts of ortho-diphenols to the system.

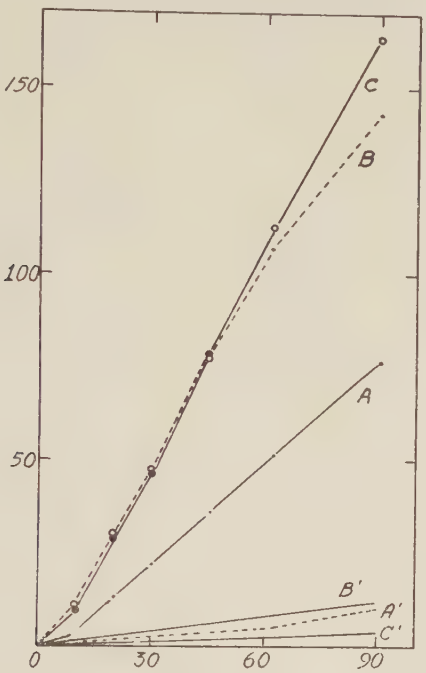

Fig. 7 The oxidation of cytochrome *c* by a particle preparation from body muscle. Cytochrome was reduced with p-phenylendiamine (curve A), hydroquinone (curve B) and ascorbic acid (curve C). Curve A′, B′ and C′: oxygen uptake in the absence of cytochrome *c*. Final concentrations in the vessels were: phosphate buffer 0.15 M; cyt. *c* 5×10^{-5} M; reducing agent 0.01 M. Abscissa: time in min.; Ordinate: μl oxygen uptake.

Several organs of *Octopus* and *Sepia* have been tested for phenolase activity. There were two main reasons for this research. First the presence of copper in all the tissues studied suggested the possibility that the enzyme was not limited only to the ink gland; second the quinone reductase activity found in several organs (see below) brought support to a postulated role of phenol oxidase in terminal respiration.

TABLE 8

Cytochrome oxidase activity of particle suspensions prepared from several organs of Octopus. (μl oxygen uptake in 1 hr.)

Final concentrations in the Warburg vessels: 0.15 M phosphate buffer pH 7.3. 0.01 M substrates; 5×10^{-5} M cytochrome *c*. 0.5 ml of the original particle suspensions were used. Gas phase: air. T = 25°C.

ORGANS	HYDROQUINONE			ASCORBIC ACID			P-PHENYLEN-DIAMINE		
		Cyt. *c*	Incr.		Cyt. *c*	Incr.		Cyt. *c*	Incr.
Kidney	17	351	334	8	300	292	4	59	55
Body muscle	8	108	100	1	114	113	5	51	46
Hepatopancreas				6	369	363	0	51	51
Branchial heart				6	25	19			
Gill				8	218	200			
Central heart	18	314	296						

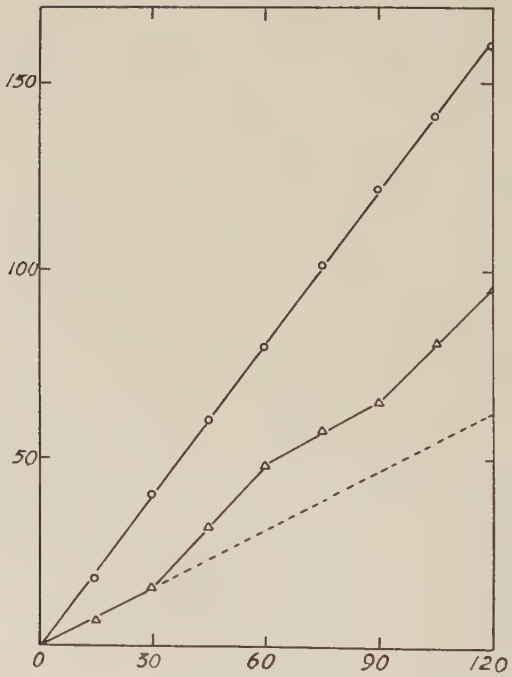

Fig. 8 The effect of alternate darkness and illumination on the oxidation of cytochrome *c* by a particle preparation from body muscle. Abscissa: time in min.; Ordinate: μl oxygen uptake.

Crude extracts prepared from hepatopancreas, kidneys, gills and muscle showed no activity towards mono-, di- and polyphenols. The oxygen consumption was never higher than that found in the controls with equivalent amounts of inorganic copper. Several copper fractions extracted from hepatopancreas according to the method described by Mann and Keilin ('39) for hepatocuprein, showed also no phenolase activity.

TABLE 9

Effect of CO : O_2 (95% : 5%) in the dark and in the light on the cytochrome oxidase activity of particle suspensions

Final concentrations: 0.15 M phosphate buffer pH 7.3; 0.01 M ascorbic acid; 5×10^{-5} M cytochrome *c*. T = 25°C.

PARTICLE SUSPENSION	N_2: O_2 O_2 UPTAKE	CO DARK		CO LIGHT	
		O_2 uptake	% inhib.	O_2 uptake	% inhib.
Muscle	40	15	62	39	0
Hepatopancreas	228	62	73	219	0

2. Phenolase activity of hemocyanin. The phenolase activity of hemocyanin has been known since 1938 when Bhagvat and Richter showed that crystalline hemocyanin from snail and crabs blood oxidizes catechol. The authors concluded that hemocyanin had a pseudo-phenolase activity comparable with the pseudo-peroxidase activity of hemoglobin.

These observations have been confirmed using pure hemocyanin prepared from Octopus blood. It was found that the protein oxidizes catechol very rapidly with the formation of a deep brown colored pigment. The oxidation of monophenols is very slow and starts only after a long induction period. The Q_{O_2} found for catechol (µl O_2 per mg protein per hr.) was higher than that found for Helix by Bhagvat and Richter. Moreover it was found that in the presence of catalytic amounts of di-orthophenols the oxidation of monophenols was greatly increased and no induction time was observed (figs. 9 and 10). The activity was strongly inhibited by cyanide and by several copper reagents. Copper-free hemocyanin pre-

pared according to Kubowitz ('38) by long dialysis against KCN had no activity towards phenols. The activity was restored after reconstitution of the protein.

3. Quinone-reductase activity of the organs. A pyridine nucleotide quinone reductase which catalyzes the reduction of para-benzoquinone has been recently purified from peas by

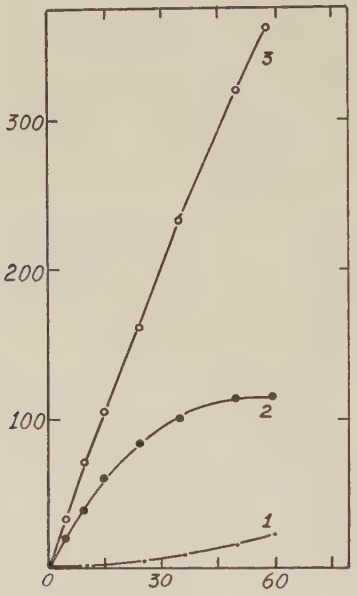

Fig. 9 The activity of *Octopus* hemocyanin on mono-, di- and polyphenols. Substrates 30 μM in 0.01 M phosphate buffer pH 7.0. Hemocyanin 8.75 mg. Abscissa: time in minutes; Ordinate: μl O_2. 1, p-cresol; 2, pyrogallol; 3, catechol.

Wosilait and Nason ('54). The enzyme was also found in a number of plants, animals (rabbit and pig) and microorganisms. By coupling the enzyme with laccase and polyphenoloxidase, it was demonstrated that the enzyme could act between quinone and pyridine nucleotides as an intermediate in electron transfer between substrate and phenolase system.

No indication exists for a similar operating system in Invertebrates.

Several organs of Octopus have been tested for quinone reductase activity. The results obtained are reported in table 10. The highest values are found in kidneys, gills and branchial hearts; they are comparable to those obtained by Wosilait and Nason for higher plants and mammals: pea seed 116, pig heart 350 and rabbit kidney 635. It must be remembered that higher values have been found only in bacteria and molds (2360–3800).

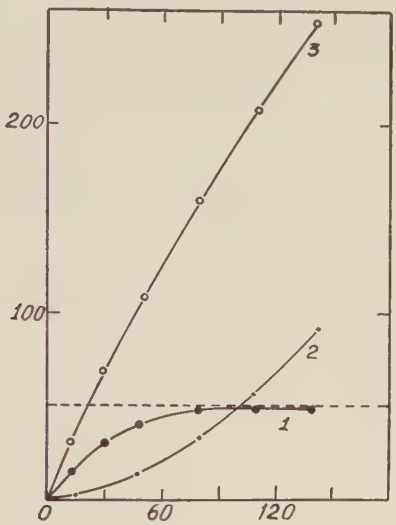

Fig. 10 The phenolase activity of *Octopus* hemocyanin. The oxidation of monophenols in presence of catalytic amounts of catechol. Hemocyanin 17.5 mg; p-cresol 30 μM; catechol 2.4 μM. 1, Catechol; 2, p-cresol; 3, catechol + p-cresol.

TABLE 10

Distribution of quinone reductase in the organs of Octopus vulgaris

The specific activity is in units per mg of protein.

ORGANS	ACTIVITY
Kidney	284
Gill	208
Branchial heart	127
Salivary gland	65
Skin	42
Body muscle	41
Optical ganglion	27
Hepatopancreas	5

DISCUSSION

In the study of the terminal respiration of Cephalopods we have applied the classical methods used for yeast and mammalian organs. However the peculiar properties of the organs and tissues used have made it necessary to examine the results obtained in conjunction with the morphological constitution and the chemical composition of each of them.

Body muscle and central heart. Of the three distinct groups into which the muscle of the arms can be divided: the central muscle bundle, the muscles of suckers and the muscles which serve as connection between them (Ballowitz, '93; Guérin, '08), only the central muscle bundle has been used in our experiments. The presence of granules, first observed by Ballowitz (1893), was confirmed by Plenck ('33) who gave a very clear picture of the sarcosomes in the protoplasmic substance.

Most of the information on the proteins of Molluscan muscle must be derived from the results of recent work on Lamellibranch adductor muscle (Hall et al., '45; Lajtha, '49; Bailey, '56). Bailey has isolated from oyster and from *Pinna nobilis* adductor muscle a crystalline globulin of myosin type, which appears to consist of about 25–30% at least of total protein. According to the same author, this globulin in its amino acid pattern is a tropomyosin. In the muscle of squid Yoshimura (quoted by Bailey) demonstrated small amounts of a water-soluble tropomyosin.

Octopus muscle has peculiar plastic properties probably due to the high viscosity of its protein components. For this reason the classical methods of preparing homogenates in a sucrose medium gave negative results and long term homogenization in a phosphate buffer medium with a Waring blendor had to be used. This fact, together with the necessity of freezing the muscle below zero, probably accounts for the loss of the Krebs cycle enzymes.

The particles isolated from body muscle are shown, by the phase contrast microscope, to be sarcosomes. They sediment from a phosphate medium 0.1 M by centrifugation at 30,000

× g. The particle suspension appears to be homogeneous and the small size of the particles is probably due to fragmentation during homogenization in the Waring blendor. However the particles give a positive reaction to vital staining with Janus green B according to the method described for mammal mitochondria. Moreover the difference spectra of a thick particle suspension show the bands of the entire cytochrome system. The iron and the copper content of body muscle, together with its content in flavins has been shown to be the lowest among the organs of the animal. The Q_{O_2} is also very low. These results are probably due to the presence in muscle of large amounts of non respiring material.

Central heart muscle differs from body muscle not only in its physiological activity but also in its chemical properties. The metabolism of squid central heart was studied in detail by Barron et al. ('53) who found that at 26°C the respiration was as high as that of rat heart. The authors also gave definite evidence that in squid heart the metabolism of acetate proceeds via the citric acid cycle; citric acid synthesis was obtained on addition of acetate and oxaloacetate to heart slices; addition of citrate caused the oxygen uptake to increase up to 200%.

The high metabolism of Cephalopod hearts has been confirmed by our experiments. The Q_{O_2} is one of the highest when compared with values obtained from other organs, and, whereas the copper content does not differ from that of body muscle, the iron content and total flavin content have been shown to be 4 and 5 times respectively greater than that of body muscle.

Branchial hearts. The branchial hearts are two deep wine-red colored bodies which drive the blood by rhythmical contractions into the gills. They have thick spongy walls into which the blood can penetrate and a thin superficial muscle layer. The spongy tissue is composed of several kinds of polyhedral cells, which contain a large colored body (Marceau, '04; Turchini, '23). Attached to each branchial heart is a compact body, the pericardial gland (also called the branchial

heart appendage) which has probably an endocrine function (Kestner, '31). Whereas in Sepia and in other Decapods this organ is distinctly separate from the branchial heart, in Octopods it is not easy to separate the two bodies and therefore all our results must be referred to both branchial heart and pericardial gland.

The function of branchial heart is not understood. Ransom (1883) first supposed a glandular function. Cuénot et al. ('13) considered them excretory organs. The red blue pigment has been extracted and purified by Fox and Updegraff ('44) and contains almost all the iron present in the organ (Unpublished experiments). Its color change according to the pH is known for a long time (Bacq and Leiner, '35).

Branchial hearts have very low succinic and cytochrome oxidase activity. The oxidation of succinate is not increased by further addition of cytochrome *c*. On the contrary, the activity of quinone reductase is relatively high.

Salivary glands. Of the two pairs of salivary glands, the anterior and the posterior, only this latter have been used in this study. They are tubular glands and secrete a very viscous liquid called saliva which has a poisonous action on the central nervous system of Crustaceans (Azzi, '18; Livon and Briot, '06). The physiological properties of the glands have been extensively studied by Bottazzi ('16, '22), Sereni ('29) and more recently by Bacq and Ghiretti ('51). Besides the poisonous substance (probably a protein) they contain large amounts of extractive amines like tyramine (Henze, '29), octopamine and 5-hydroxytryptamine (Erspamer, '48a, '48b). The amount of free amino acids reaches a value of 2 gm per 100 gm of tissue (Bacq, personal communication). The amino acid and amine oxidase activity of the organs is very high (Blaschko, '52; Blaschko and Hawkins, '51).

Our results indicate that the posterior salivary gland is the site of active metabolic functions. It was not possible by any method to prepare homogenates of the glands and isolated particle suspensions, and therefore we could not obtain difference spectra of the cytochrome system. However exper-

iments on the inhibition of tissue slices respiration by cyanide and carbon monoxide, both in the dark and light, clearly indicated that the terminal oxidation of the posterior salivary glands goes through the cytochrome-cytochrome oxidase system.

Kidneys. In Octopods these excretory organs (also called venous appendages) consist of hollow branching cavities covered with a glandular epithelium. The cavities are continuous with the veins from which they hang and the epithelium is excretory in function. The organs extract waste products from the blood and pass them into the renal sac (Turchini, '23).

As mentioned already, Octopus kidney was found full of Dycyemids. All our results must therefore be considered as being contaminated with microorganisms. However, if Dycyemids do not contain iron (Emanuel and Martin, '56), then the value found for this element can be considered as correct.

Octopus kidney is the richest organ in flavin of the animal. However the FAD content if calculated in per cent of the total flavins, is relatively small. This can be an indication that the organ is probably the place of riboflavin synthesis. Moreover we must remember that the organ, like the salivary glands and hepatopancreas, has a very high oxidase activity towards amino acids (Blaschko, '52; Blaschko and Hawkins, '51).

Hepatopancreas. The organ which is called hepatopancreas consists in Octopods of a large digestive gland which fills the anterior half of the visceral dome. It is often referred to as the liver of the animal but its morphological structure as well as its function have little connection with the liver of Vertebrates.

Hepatopancreas has a very soft, almost liquid texture and its color varies from red orange to brown. The study of its fine structure shows several kinds of cells which were originally described by Enriques ('02). They contain pigmented particles of different size which are of food origin. Bidder ('50, '56) who made a very extensive study of the digestive mechanism of the Cephalopods, demonstrated that whereas

in the squid food does not enter the liver, in *Sepia* and *Octopus* it enters as a very fine suspension. In *Octopus* therefore absorption preceeds digestion as in certain microorganisms, and the hepatopancreas has both absorptive and secretory functions (secretion of digestive enzymes: see Romijn, '35). This explains the peculiar properties of a particle suspension prepared with the usual methods from the organ. The particle suspension is not homogeneous and further centrifugation in a density gradient of sucrose shows very distinctly separated layers of granules of different size and color. Whereas the cytochrome oxidase activity of the total particle suspension is very high, no oxidation of succinic acid is found. Oxidation of succinic acid could be demonstrated after centrifugation in a density gradient by the bottom layer which therefore must be considered to consist of mitochondria. The enzymatic properties of these granules as well as the chemical study of the granules in the other layers requires more study.

The hepatopancreas is the richest organ in iron and copper. Its high copper content is an indication that the organ stores this metal and that it is probably related with the biosynthesis of hemocyanin. Henze ('01) first determined copper in Cephalopods liver. The values he found are from two to three times higher than those found by us. He did not perfuse the animal with sea water and therefore we think that hemocyanin contaminated his results.

As for kidneys, the high flavin content of the hepatopancreas is in agreement with the oxidase activity towards amino acids and amines. Octopus liver readily oxidizes tyramine and 5-hydroxy-tryptamine and inactivates them (Erspamer and Ghiretti, '51). Beside d- and l-amino acid oxidases, the organ contains a specific d-glutamic acid oxidase (Blaschko and Hawkins, '52; Blaschko and Himms, '55). We have found that these oxidases are flavin enzymes (Unpublished experiments). The respiration of hepatopancreas is only 52% inhibited by cyanide, a further indication that part of its terminal respiration goes through the flavin system.

Gills and branchial glands. Gills have a very complicated pinnate structure (Joubin, 1885) which makes the application of the usual experimental methods very difficult. The branchial glands are more easy to handle. These glands lie along the dorsal side of the axes of the gills and have a very rich blood supply. These organs have been considered ductless glands, but their function is unknown (see Hutchinson, '28; Sereni, '32; Mitolo, '38).

Both gills and branchial glands have a very high metabolism as demonstrated by their Q_{O_2}, their flavin content and enzymatic activity. The high quinone-reductase activity of the organs seems to be an indication that, even if the main route of terminal respiration is represented by the cytochrome system (as clearly demonstrated by inhibition of the respiration and by the cytochrome oxidase activity), there exists the possibility of other pathways.

CONCLUSIONS

As mentioned in the introduction, our experiments on the respiratory systems of Cephalopods started from the possibility that animals which have in the blood a pigment different from hemoglobin as oxygen carrier, might have also respiratory enzymes which are different from iron porphyrin compounds. Cephalopods were taken as experimental animals because they are the most active and organized animals among marine Invertebrates. Octopus removes 70–80% of the oxygen from incoming water, a level which is reached only by certain fishes and which is by far higher than the oxygen utilization of Crustaceans, Lamellibranchs and Tunicates (Winterstein, '09; Hazelhof, '39). Moreover it has been clearly demonstrated that in Cephalopods hemocyanin is the oxygen carrier of the blood (Wolvekamp, '37; Redfield, '33; Henze, '01). The hemocyanin content of the blood of such active animals as *Octopus, Loligo* and *Sepia* is much higher than that of more sluggish forms and is correlated therefore with the degree of their oxygen utilization.

The first experimental proof that the terminal respiration in Cephalopods goes through iron enzymes is given by the results of our experiments on the effect of carbon monoxide. Carbon monoxide was selected among the inhibitors as the most specific agent by which the participation of iron or copper enzymes could be selectively demonstrated. It is well known that the reversible inhibition of respiration by carbon monoxide indicates that the terminal oxidases are heavy metal catalysts. If the carbon monoxide inhibition is light sensitive, then iron is the heavy metal. This rule was established by Warburg in 1926 and no exception has been found to it. In general the organs of Cephalopods cannot be used with great success for these inhibition studies for several reasons: first the pigments present in many of them (hepatopancreas, kidney, branchial heart) are a great hindrance to light penetration; secondly, the Q_{O_2} of the organs which, already low in air, is further reduced in the experimental conditions required for CO inhibition.

The most satisfactory results were obtained using salivary glands. The respiration of thin slices prepared from the organs was found to be very sensitive to carbon monoxide in the dark. Moreover the inhibition was totally reversed by light. Several attempts were also made with other organs, but for the above mentioned limitations, the results were not as clear as for salivary glands. Nevertheless the behavior of these organs towards the inhibitor indicated that in all of them, iron-porphyrin compounds and not copper oxidases are the catalysts of terminal respiration.

Cyanide is known to be a strong inhibitor of metal enzymes. A respiration which is partly insensitive to cyanide ("residual respiration") is assumed to go through flavins (Commoner, '40). Our results on the effect of cyanide on tissue respiration of Cephalopods give a further demonstration that in most of the organs studied terminal respiration is catalysed by metallo enzymes. Moreover the results show that direct oxidation of flavins do not participate in terminal electron transfer. The only exception is given by the hepatopancreas where almost 50% of respiration is not sensitive to cyanide.

Flavins have been detected in Insects (Bodine and Fitzgerald, '47; Busnel and Drilhon, '42; De Lerma, '49) and in several other Invertebrates (Gourewitch, '37). A quantitative study of riboflavin in Tunicates was made recently by Fish et al. ('51). Our results indicate that Cephalopod organs contain amounts of riboflavin, FMN and FAD comparable to those of Mammals. The high flavin content of the organs must be related to the great variety of amino acid and amine oxidases of the organs. Cephalopods are ammonothelic animals (Delaunay, '25, '34) and the great amounts of free amines and amino acids in the tissues, together with the corresponding oxidases, suggest that these compounds play an important metabolic role in these organisms.

Spectrophotometric studies on particles isolated from the tissues demonstrate that the iron respiratory enzymes present, are cytochromes. This cytochrome system is formed by cytochrome *b*, *c*, *a* and a_3 and it is operating in the terminal electron transfer to molecular oxygen.

The presence of hematin compounds in organisms with a blood oxygen pigment different from hemoglobin, was first indicated in 1886 by Mac Munn. The pigment he found in muscles and in other organs of animals from almost all classes of the animal kingdom was called myohematin or histohematin and its respiratory function was recognized on the basis of its ability to undergo reversible reduction and oxidation. Against Hoppe-Seyler's objection that myohematin was an hemochromogen derived from hemoglobin, Mac Munn emphasized the presence of the pigment also in those animals without hemoglobin: "Zum schlusse will ich noch einmal die Behauptung aufstellen dass Histohematine im allgemeinen bei Tiere vorkommen bei denen sich keine Spur von Haemoglobin oder Haemocromogen erkennen lasst." Among these animals were Molluscs and Arthropods with hemocyanin.

It is well known that Mac Munn's myohematin was rediscovered by Keilin in 1925 and was called Cytochrome. According to Keilin, cytochrome for its wide distribution must be considered the most ancient respiratory pigment, perhaps

the first to be formed during evolution of living organisms and from which all other iron porphyrin respiratory pigments have been derived.

Since these classical studies, very little attention has been paid to the study of electron transfer in the last steps of terminal respiration in marine organisms possessing hemocyanin. We may mention the work of Ball and B. Meyerhof ('40) who, by spectroscopic observations, detected in thin slices of heart muscle of several Molluscs (*Busycon, Loligo, Venus*) and of *Homarus* and *Limulus* the absorption bands of cytochromes *a, b* and *c*. More recently, by microspectrophotometric methods, Cooperstein and Lazarow ('51) studied the cytochrome oxidase and succinic dehydrogenase content of the squid nervous system; succinic dehydrogenase activity was also found by Nachmansohn et al. ('42, '43) in the giant axon of the squid.

The difference spectra of the particle suspensions prepared from the body muscle and other organs show that the cytochrome system of Cephalopods is very similar to that of Mammals. Distinct peaks in the Soret and the visible regions can be seen which correspond to the γ, β and α bands of the cytochromes *a*, a_3 and *b*. The bands of cytochrome *c* are not visible in these spectra. However cytochrome *c* is present in the material as indicated by the succinic oxidase activity of the particle suspensions. Cytochrome *c* in the particle preparations is certainly less abundant than the other cytochromes and this might well be due to loss of this water soluble component during the several prolonged washings of the tissues with water. It must be remembered that in general it is difficult to detect γ band of cytochrome *c* in difference spectra. Difference spectra from bacteria (Smith, '54) and from heart muscle and *Acetobacter pasterianum* (Chance, '52) all of which contain cytochrome *c*, do not show the γ band of this pigment. Even the difference spectra of rat liver mitochondria observed with a very sensitive instrument, show only a very faint γ band of cytochrome *c* (Chance and Williams, '56). This difficulty is probably related to the fact that the band

of reduced cytochrome *c* is very near to the Soret band of oxidized cytochrome *b* and can therefore be cancelled by it in the difference spectra.

The spectral study of the CO compound with cytochrome a_3 gives further identification of cytochrome oxidase in Cephalopods. Both the peaks at 430 mμ and the trough at 445 mμ are characteristic of the mammal enzyme. It must be noted that old determinations of photochemical spectra by Melnick ('45) do not agree with these values, but they are in the very same position to the corresponding values found by Chance ('53) for heart muscle particles from pig heart and from yeast.

Finally the increment of the oxidation of ascorbic acid and other substrates found after addition of cytochrome *c* and the photoreversible inhibition of this increment by carbon monoxide, are indirect evidence for the presence in Octopus tissues of a cytochrome oxidase similar to mammal and yeast enzyme.

With the demonstration of an operating cytochrome system in Cephalopods, it is clear that the terminal electron transfer between organisms having hemocyanin as blood oxygen carrier is the same as in animals with hemoglobin. As Keilin pointed out in 1925: *"the mere presence of a hemoglobin in an organism does not necessarily imply the existence of a deep physiological difference between this organism and another which is devoid of this pigment."*

SUMMARY

1. The pathway of terminal respiration in *Octopus vulgaris, O. macropus* and *Eledone moschata* has been studied using intact tissues and particles isolated from several organs. A method of preparing particle suspensions from body muscle and hepatopancreas by differential centrifugation is described. The particle suspensions obtained, which give a positive reaction to vital staining with Janus green B, have been used for spectrophotometric and enzymatic activity determinations.

2. The iron, copper and flavin content has been determined in a number of organs. Hepatopancreas is the richest organ in iron and copper. The highest levels of flavins (determined as riboflavin, FMN and FAD) have been found in kidney and hepatopancreas.

3. Manometric determinations of the oxygen uptake of tissue slices show that the organs of Octopods are very sensitive to the partial oxygen pressure. The respiration of most of the organs studied is more than doubled at a higher partial oxygen pressure. Sensitivity to cyanide has been found to be very high for all the organs and tissues; 80–100% inhibition is obtained with 10^{-3} M KCN. The only exception is the hepatopancreas where the inhibition is only 50%. Carbon monoxide inhibits the respiration of tissue slices from 19 to 49%. This inhibition is entirely reversed by light. These experiments clearly indicate that terminal respiration in Cephalopods is catalysed by iron enzymes.

4. Difference spectra taken on isolated particle suspensions show the presence of a complete cytochrome system formed by cytochrome *a*, a_3, *b* and *c*. As indicated by the peaks of the observed bands, this cytochrome system is very similar to that of Mammals and yeast. Cytochrome a_3 has been also identified by the difference spectrum of its compound with CO.

5. The succinic oxidase and cytochrome oxidase activity of the isolated particles has been studied. Addition of mammal cytochrome *c* increases the oxidation of succinate. The cytochrome oxidase activity of the particles is strongly inhibited by CO and the inhibition is totally abolished by light.

6. No indication has been found for the existence and the participation in the terminal respiration of copper enzymes. Beside the ink gland, only pure hemocyanin prepared from the blood has been found to possess phenolase activity. Even copper proteins extracted from hepatopancreas and partially purified have no enzymatic activity. A DPN dependent quinone reductase has been found in several organs. The possibility of a coupling of this enzyme with phenolase and its role in terminal respiration of Cephalopods can be excluded

on the basis of carbon monoxide inhibition of tissue respiration.

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THE EFFECTS OF NITROGEN, HELIUM, ARGON AND SULFUR HEXAFLUORIDE ON THE DEVELOPMENT OF INSECTS[1]

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FOUR FIGURES

INTRODUCTION

The physiological effects of helium and argon, at atmospheric pressure, have received little study, since both of these gases have been considered physiologically inert. However, in 1950, Cook reported that helium, and to a lesser extent argon, when substituted for the nitrogen in the atmosphere, significantly accelerated the development of two insects, the mealworm, *Tenebrio molitor,* and *Drosophila melanogaster.* He offered no hypothesis to explain this surprising result. The present experiments were designed to confirm and extend these findings on the effects on insect development of helium and argon at atmospheric pressure. In addition a study was made of the action of positive pressures of these and other physiologically inert gases.

In contrast to the paucity of information on the action of gases at atmospheric pressures, the physiological effects of gases at high pressures have been studied extensively and a comprehensive bibliography has been prepared by Hoff ('48). Early work by Meyer and Hopff ('23) showed that nitrogen at a pressure of 90 atmospheres produced reversible narcosis in

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amphibians. In humans, Behnke and his co-workers ('35) found that breathing air at a pressure of three atmospheres slowed mental activity and impaired neuromuscular coordination. A mixture of helium and oxygen at similar pressures eliminated these depressant effects (Behnke and Yarbrough, '38). Argon, by contrast, induced a greater stupefaction and neuromuscular impairment than did air. Behnke and Yarbrough ('39) concluded that their results were attributable to nitrogen and argon narcosis. These effects were in turn correlated with the respective oil-water coefficients of these gases: 1.7 to 1 for helium, and 5.3 to 1 for both nitrogen and argon. Argon was a more effective narcotic than nitrogen presumably because it has twice the *absolute* solubility in oil.

Carpenter ('53) attempted to quantify the narcotic actions of nitrogen, argon, nitrous oxide and sulfur hexafluoride, by determining that partial pressure of the gas which, when superimposed on an atmosphere of pure oxygen, would protect 50 % of the animals tested (mice) from maximal electroshock convulsions. The values he found were as follows: N_2O: 8.6 psi; SF_6: 27.5 psi; A: 185 psi; N_2: 265 psi. It is significant in this connection, that SF_6 has an oil-water partition coefficient of about 200: 1 (Tenney, Carpenter and Rahn, '53). Marshall ('51) found that preparations of isolated sciatic nerve, as well as nerve-muscle preparations, were unaffected by nitrogen, even at pressures up to 96 atmospheres. Reflex activity of the spinal cord, however, could be blocked by nitrogen at 17 atmospheres, or by argon at 10 atmospheres. Helium, on the other hand, had no effect at pressures up to 82 atmospheres.

In insects, Meyer and Hopff ('23) demonstrated that 90 atmospheres of nitrogen reversibly narcotized the cockroach *Blatta orientalis*. Chadwick and Williams ('49) found that *Drosophila* was active at 5 atmospheres of nitrogen and one atmosphere of air, and Williams (unpublished) found that 7 hours of exposure to 24 atmospheres of nitrogen plus one atmosphere of air failed to affect the vitality of *Drosophila* upon subsequent return to air. Likewise in the Cecropia silk-

worm at all stages of development from egg to adult, prolonged exposure to 6.7 atmospheres of nitrogen plus one atmosphere of air caused no conspicuous inhibition of respiration, embryonic or adult development, heart-beat, movement, or the spinning of the cocoon (Schneiderman and Williams, '54a, b).

In 1955, in connection with a study of oxygen poisoning, Goldsmith observed that nitrogen at 5 and 10 atmospheres often exerted a slight narcotic effect on the chalcid wasp *Mormoniella.* She noted also that pupae of this insect after exposure to 5 atmospheres of helium respired at a more rapid rate than those which had been exposed to an equal pressure of nitrogen. This was the first report of the narcotic effects on insects of a physiologically inert gas at modest pressures, and it prompted the present study. Since little was known of the effect on insects of gases at high pressures, we examined the effects on *Mormoniella* of positive pressures of 4 gases with widely different oil-water partition coefficients and absolute solubilities: nitrogen, helium, argon and sulfur hexafluoride (Lawrence et al., '46).

MATERIALS AND METHODS

Experiments at atmospheric pressure were performed on embryos, larvae, pupae, developing adults and adults of the mealworm, *Tenebrio molitor* and of the chalcid wasp, *Mormoniella vitripennis* (Walker). Experiments at positive pressures utilized only *Mormoniella.* This wasp which is a parasite of dipterous pupae was raised on puparia of the flesh-fly, *Sarcophaga bullata,* according to the technique of Whiting ('55). Its life cycle has been described in detail by Tiegs ('22) and by Whiting and is considered only briefly here. The female *Mormoniella* lays her eggs within the fly puparium. When the larvae hatch they feed on the fly pupa and molt 4 times. At 25°C, about 6 days after the eggs have been laid the mature larva ceases feeding and enters a "resting stage." Within the next 36 hours the breakdown of larval tissues and proliferation of the imaginal discs begins. The resting stage terminates with defecation, which occurs when cell division leads to a break-

down of the thin partition between the mid-gut and the invaginated rectum. About 20 hours after defecation a pupal cuticle is secreted and the pupal molt occurs. The pupa is at first white and unpigmented, but becomes pink after a few hours. Within a day the pupal cuticle detaches from the epidermis and adult development begins. The first external sign is eye pigmentation which proceeds through a pink, red and finally a dark red-brown stage. Shortly after this last stage is reached, body pigmentation commences, first in the head and thorax, and then in the abdomen. During this period of adult development, which lasts about 4 days at 25°C, the appendages, integument, nervous system, etc., which had proliferated at the end of the last larval instar, differentiate into their final adult form. On the fifth day the black developing adult stage is reached and the fully differentiated and pigmented adult lies enclosed within the transparent pupal cuticle. About a day later the adult resorbs its molting fluid and emerges.

Gas mixtures

All gases used in the experiments, except for the air from the laboratory outlet, were from commercial cylinders (Airco or Matheson) and assayed better than 99% pure. Gas mixtures were prepared under pressure and stored in 4-liter stainless steel cylinders and their compositions checked by gas analysis (Scholander, '42). In all cases, percentage compositions were accurate to $\pm$ 1 per cent. The methods utilized in management of the various gas mixtures were simple.

Experiments at atmospheric pressure

In experiments with *Tenebrio* at atmospheric pressure a continuous flow system essentially like that of Cook ('50) was employed (fig. 1). An appropriate gas mixture was run through a flask containing animals and food. Frequent gas samples were taken from the outlet tube and analyzed to insure a flow rate rapid enough to prevent the accumulation of carbon dioxide. In experiments with *Mormoniella* at atmospheric

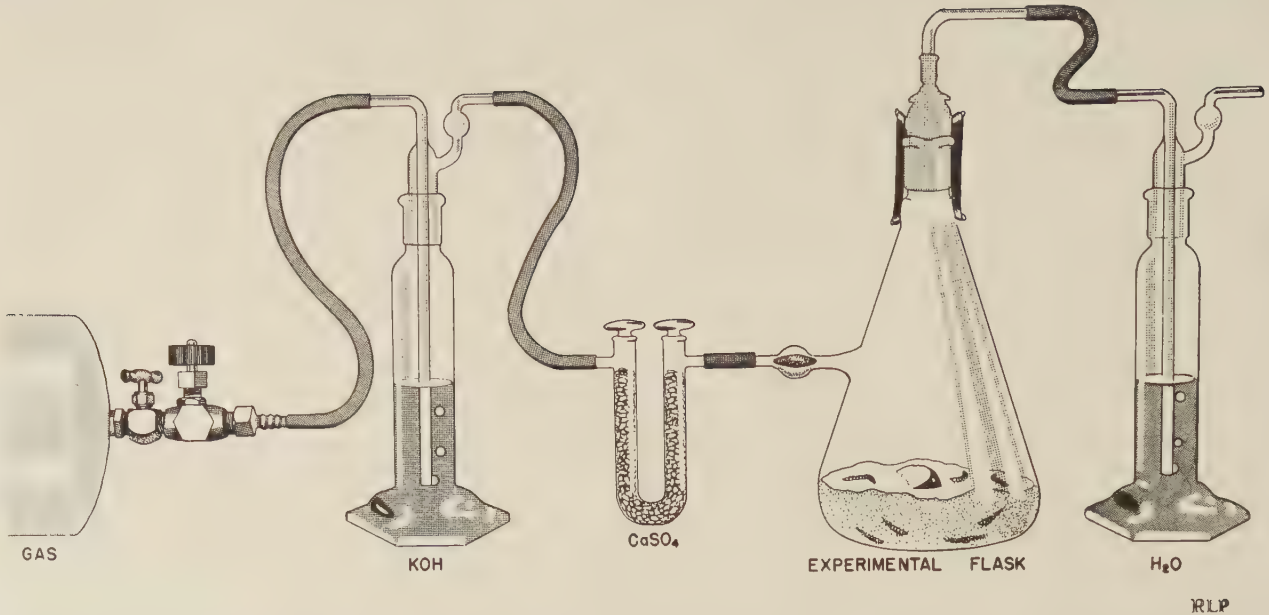

Fig. 1 Diagram of continuous flow system used in experiments with *Tenebrio*. One hundred animals at a specific stage were selected and placed together with 100 gm of dry bran and 25 gm of apple, for moisture, in the bottom of a 2-liter Ehrlenmeyer flask fitted with a standard taper ground glass joint and aside arm. From a steel cylinder fitted with two needle valves for fine adjustment of rate of flow, the desired gas mixture was passed through a KOH solution to absorb carbon dioxide and then through $CaSO_4$ to remove the excess moisture. The gas was then run into the experimental flasks through the side arm, out through the glass joint top and through a water trap. The rate of flow of gases was regulated to keep the carbon dioxide concentration in the flasks close to zero.

pressure animals were placed in large (3.5 liter) or in small (0.66 liter) transparent polymethyl methacrylate (Lucite) chambers fitted with brass end-plates and needle valves (Schneiderman and Feder, '54). Following this a mixture of 21% oxygen and 79% of the appropriate gas was freed of carbon dioxide, humidified and a large volume flushed through the chamber to wash out the residual air. The chambers were then sealed and stored at constant temperature. In addition periodic reflushing was performed at regular intervals to maintain the oxygen tension at 21% and to prevent any accumulation of carbon dioxide.

In studies of the larval-pupal and pupal-adult transformation, *Mormoniella* were removed from the fly puparia and placed on corrugated cardboard platforms in such a way that the development of several hundred animals could be observed individually. The platforms were then slipped into the small Lucite chambers, gas mixtures run through and the chambers sealed as described above. Periodic observations were made through the transparent walls of the chamber until all the adults had emerged.

When the development of the whole life cycle was studied at atmospheric pressure, *Sarcophaga* puparia which had been parasitized by *Mormoniella* in the previous 24 hours were sealed within the large Lucite chambers. The desired gas mixtures were flushed through at the start of the experiments, and re-flushed at 4-day intervals. At intervals a series of chambers were opened, the *Sarcophaga* puparia removed and broken open and a record made of the development of the wasps. Observations were continued periodically until all the animals had emerged as adults.

Experiments at positive pressure

In experiments at positive pressure the chambers were initially flushed as described above with a mixture of 21% oxygen and 79% of the appropriate gas. Following this the chambers were compressed with either 5 or 10 atmospheres of

the specific gas. At the completion of this maneuver, the oxygen tension in the chambers was that of air, namely 21% of an atmosphere, while the pressure of the added gas was the gauge pressure plus 79% of an atmosphere. Compression was gradual, proceeding at the rate of about one atmosphere per minute. However, Goldsmith ('55) has demonstrated that pressure *per se* has no adverse effects on *Mormoniella* and that even instant compression and decompression fail to affect developing larvae, pupae, developing adults, and adults. All of the positive pressure experiments were performed at 25°C.

When larvae, pupae, and developing adults were used they were removed from the fly puparia and their development followed individually on corrugated cardboard platforms within the small 0.66 liter Lucite chambers. In these experiments at positive pressures no more than 80 wasps were enclosed in each chamber. Since the oxygen uptake of these few wasps was small it did not materially diminish the oxygen concentration within the chambers and periodic flushing was unnecessary.

For studies of the whole life cycle, recently parasitized puparia containing many hundreds of developing wasps were sealed and compressed within large 3.5 liter Lucite chambers. Such a procedure made periodic flushing difficult. Instead, calculated volumes of oxygen were periodically added to replace the oxygen consumed by the *Mormoniella* and the fly pupae. In some experiments vials of potassium hydroxide were placed within the chambers to absorb carbon dioxide, but identical results were obtained in experiments where no potassium hydroxide was present. After storage at 25°C for a specific period, the chambers were gradually decompressed, the puparia opened and the developmental stage of the wasps recorded.

The pressure of each chamber was checked daily to detect leaks. In all experiments reported here, pressure never varied more than $\pm$ 0.3 atmospheres during the entire course of an experiment.

RESULTS

Effects of helium and argon at atmospheric pressure on Tenebrio and Mormoniella

A large series of experiments was performed involving many thousands of mealworms and wasps at various stages of development. Great care was taken to regulate gas flow, moisture, carbon dioxide accumulation, light, as well as to make a strictly random initial selection of animals. Experiments were conducted at 22°C, the temperature used by Cook ('50) as well as at 20°C, 25°C and 30°C. The results were wholly negative (Frankel, '55). Under the conditions of our experiments helium and argon when substituted for the nitrogen in the atmosphere failed to affect the rate of development of *Tenebrio* or *Mormoniella* at any stage and at any of the temperatures studied. On the basis of these findings we concluded that, contrary to earlier reports, inert gases at atmospheric pressure do not accelerate the development of insects.[3] Our attention next turned to positive pressures of these gases.

Effects of nitrogen, helium and argon at elevated pressures on Mormoniella

Effects on adult development. In two series of experiments summarized in table 1, *Mormoniella* pupae were compressed with nitrogen, helium and argon to a pressure of 5 atmospheres. No significant differences were found in the rate of adult development or the time required for black developing adults to emerge between any of the gases, nor between the gases and air at atmospheric pressure. It does not appear that 5 atmospheres of nitrogen, helium or argon had any effect on the rate of adult development or on emergence.

In a second series of experiments also recorded in table 1 pupae were compressed to 10 atmospheres with the several gases. Helium at this pressure still failed to exert any appreciable delaying effect compared to controls in air at atmospheric

[3] We wish to thank Professor S. F. Cook for his helpful comments on the results of these experiments.

TABLE 1

The effects of 5 and 10 atmospheres of nitrogen, helium and argon on the rate of adult development of Mormoniella at 25°C

1	2	3			4			5(4 − 3)		
PRESSURE (ATM.)	STAGE OF DEVELOPMENT AT OUTSET IN DAYS AFTER PUPATION	NUMBER OF DAYS TO COMPLETE ADULT DEVELOPMENT (± S.D.)			TOTAL NUMBER OF DAYS TO COMPLETE ADULT DEVELOPMENT AND EMERGE (± S.D.)			NUMBER OF DAYS FOR ADULT EMERGENCE		
		Air + N_2	Air + He	Air + A	Air + N_2	Air + He	Air + A	Air + N_2	Air + He	Air + A
5	1 (pink pupa)	3.5	3.4	3.4	5.0	4.9	5.1	1.5	1.5	1.7
5	0.5 (white-pink pupa)	4.0	4.0	3.9	5.3	5.3	5.3	1.3	1.3	1.4
5	last instar larva	5.9	5.4	5.8	7.7	6.7	7.7	1.8	1.3	1.9
10	1 (pink pupa)	4.2 ± .42	3.7 ± .42	4.1 ± .55	6.5 ± .55	5.5 ± .44	6.5 ± .52	2.3	1.8	2.4
10	1 (pink pupa)	4.4 ± .41	4.1 ± .53	4.6 ± .54	6.7 ± .53	5.8 ± .55	7.0 ± .66	2.3	1.7	2.4
10	3.5 (red-brown eye developing adult)	—	—	—	—	—	—	1.5	—	1.5

Each figure is based on 72 individuals (less a small number which died because of handling).

The average time of development of pink pupae in air was 3.8 days; the average time of emergence was 1.6 days.

Standard deviations were obtained graphically by plotting the data on "probit" paper. Where standard deviations are not given, the animals reached the stated stage within one day of each other, so that no standard deviations could be calculated.

pressure. Argon and nitrogen, however, caused a significant and consistent delay in adult development, and a still more striking delay in the time required for black developing adults to emerge. Furthermore, argon (but not helium or nitrogen) prevented the complete emergence of 30 to 40% of all the animals observed. The inhibited animals emerged only partially and, failing to fully free themselves from their pupal cuticles, became motionless after a day or two. Even among those animals which emerged fully, argon (and nitrogen to a lesser extent) delayed and often prevented full expansion of the wings.

Further experiments demonstrated that the adverse effects of 10 atmospheres of argon and nitrogen were the cumulative result of continued exposure to these gases. When fully differentiated black developing adults were compressed with 10 atmospheres of argon or nitrogen, all the wasps emerged promptly. When developing adults at an intermediate stage were so compressed, there was a relatively short delay in adult development (see table 1), and argon prevented only 20% rather than 30 to 40% from emerging.

Effects on the whole life cycle. Table 2 summarizes three experiments which appraised the effects of compression to 5 atmospheres on the whole life cycle. In all three experiments the animals kept in argon and nitrogen took significantly longer to develop than did those kept in helium, the average delay amounting to about one day. Argon and nitrogen both had about the same delaying effect. These delaying effects were apparently exerted exclusively on the larva, since the previous experiment showed that neither argon or nitrogen at 5 atmospheres had any effect on the rate of the pupal-adult transformation.

In the first experiment in table 2, a control in air at atmospheric pressure was included. These animals developed about half a day ahead of those kept in helium at 5 atmospheres. Possibly 5 atmospheres of helium had a slight narcotizing effect. But in our opinion it is more likely that pressure *per se* had some retarding effect on development, probably on the larval stages, although the data do not distinguish between these

TABLE 2

The effects of 5 atmospheres of nitrogen, helium and argon on the rate of development of Mormoniella during the whole life cycle

GAS MIXTURE	PER CENT AT VARIOUS STAGES OF DEVELOPMENT							
	Air		Nitrogen-Oxygen		Helium-Oxygen		Argon-Oxygen	
DAYS OF DEVELOPMENT	15	16	15	16	15	16	15	16
Stage of development:								
Last instar larva	0	0	7	0	0	0	15	0
Developing adult not yet black	19	11	66	54	40	24	79	77
Black developing adult	39	8	20	18	31	16	6	17
Emerging adult	2	2	1	2	1	3	0	2
Adult	40	79	6	26	28	57	0	4
Total number of animals	770		426		596		582	
Black developing adult			53	13	24	1	57	6
Emerging adult			4	3	6	0	4	2
Adult			43	84	70	99	39	92
Total number of animals			600		264		549	
DAYS OF DEVELOPMENT			16.5	17.5	16.5	17.5	16.5	17.5
Stage of development:								
Developing adult not yet black			44	31	4	0	29	13
Black developing adult			41	20	17	9	53	25
Emerging adult			3	2	1	2	3	5
Adult			12	47	78	89	15	57
Total number of animals			323		397		664	

possibilities. However, this slight effect of helium or pressure was not nearly so great as the delaying effects of argon and nitrogen.

Effects on mature larvae. Mature larvae just prior to defecation were exposed to five atmospheres of helium, argon and nitrogen until they emerged as adults. The results, summarized in table 1, reveal that the animals kept in argon and nitrogen took half a day longer to develop into black developing adults and the black developing adults took half a day longer to emerge, than did those compressed in helium. Further analysis of the day-by-day data revealed that the delay in development to the black developing adult stage was confined entirely to the larval-pupal transformation; adult development itself was not affected.

It is therefore likely that most or perhaps all of the delaying effect on development induced by 5 atmospheres of argon and nitrogen is exerted specifically on the last larval instar, particularly during the proliferation of imaginal tissues and the formation of the pupa. The delay in adult emergence induced by continuous exposure to the gases from the mature larva onward, is probably the result of damage that occurred during the last larval instar, since no such delay resulted when pupae were exposed to 5 atmospheres of these gases (table 1).

Effects of sulfur hexafluoride at atmospheric pressure on Mormoniella

Effects on adult development. Pupae exposed for the entire period of adult development to a mixture of 21% oxygen and 79% sulfur hexafluoride invariably took about 50 per cent longer to develop than did the air controls. About one-third of these pupae ceased developing before the black developing adult stage and those that succeeded in becoming black developing adults never began adult emergence.

In further experiments groups of pink pupae were exposed to mixtures of sulfur hexafluoride and oxygen for varying periods, after which the chambers containing the animals were thoroughly flushed with air. The results are recorded in figure

2. It can clearly be seen that the effect of sulfur hexafluoride on the ability of adults to emerge is a cumulative one: pupae exposed for short periods emerged regularly while pupae exposed for 6 days failed to emerge no matter how long the subsequent exposure to air.

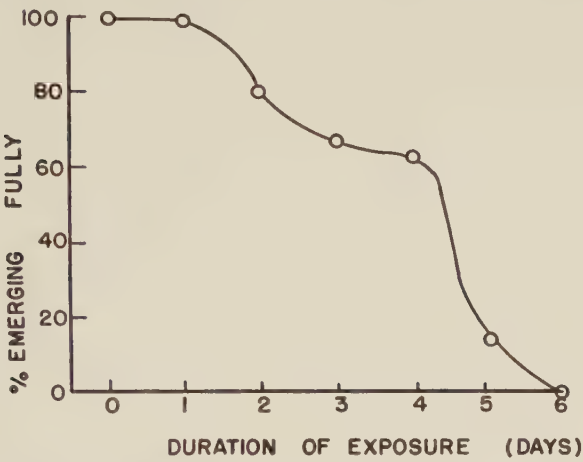

Fig. 2 The effect of varying periods of exposure to SF_6–O_2 mixtures on the adult emergence of *Mormoniella*. The abscissa represents the total period of exposure, in days, from the beginning of adult development; the ordinate shows the per cent of animals which emerged fully.

In further experiments which are summarized in figure 3, animals at different stages of adult development were exposed for one day to sulfur hexafluoride. It appears from the results that if this period of exposure was confined to any stage in adult development before the terminal one, normal emergence eventually took place. However, if exposure occurred during the period when the fully differentiated black developing adult was preparing to emerge, then one day of exposure almost completely inhibited emergence.

Effects on adult behavior. Exposing normal active adult *Mormoniella* to mixtures of sulfur hexafluoride and oxygen for one day caused irreversible paralysis. In some individuals the paralysis was not complete until 12 hours after the wasps

were returned to air. The oxygen uptake of the paralyzed wasps was measured in Warburg respirometers. Although their uptake was lower than that of either normal active adults or of black developing adults, it was substantial enough to show that these animals were merely paralyzed but not dead. Twelve hours of exposure left the animals still active and apparently unharmed.

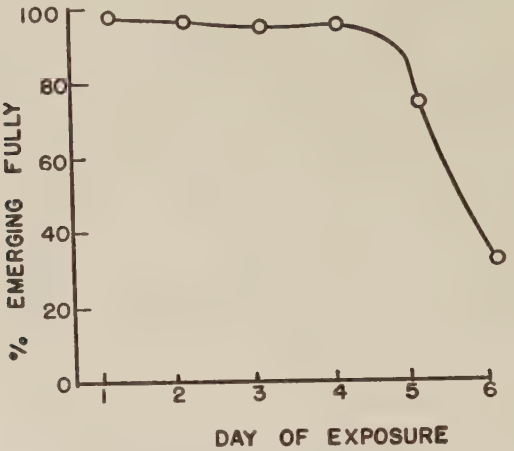

Fig. 3 The effect on adult emergence of one day of exposure to SF_6–O_2 mixtures at various stages of adult development. The abscissa represents the day of development during which developing adults were exposed; the ordinate marks the per cent of animals which emerged fully.

Effects on eggs and young larvae. When newly parasitized puparia of *Sarcophaga* were opened after 12 days of exposure to mixtures of SF_6 and oxygen, they occasionally contained a few, tiny *Mormoniella* larvae, indicating that a considerable amount of cell division had taken place in the presence of SF_6.

Effects on mature larvae. Larvae just prior to defecation were exposed to mixtures of SF_6 and oxygen for specific periods. The results presented in figure 4 show that SF_6 generally did not inhibit defecation, indicating that the cell divisions involved in breaking the partition between the midgut and hindgut occurred in the presence of SF_6.

Sulfur hexafluoride had a much more profound effect on pupation. Exposure of mature larvae for 36 hours or more commonly inhibited normal pupation. Those larvae which succeeded in defecating only pupated partially. Their body regions became vaguely distinguished and the wing and limb anlagen partially everted, yielding abnormal pupae which resembled, superficially at least, the abnormal pupae produced by x-rays (Schneiderman, Kuten and Horwitz, '56). Such

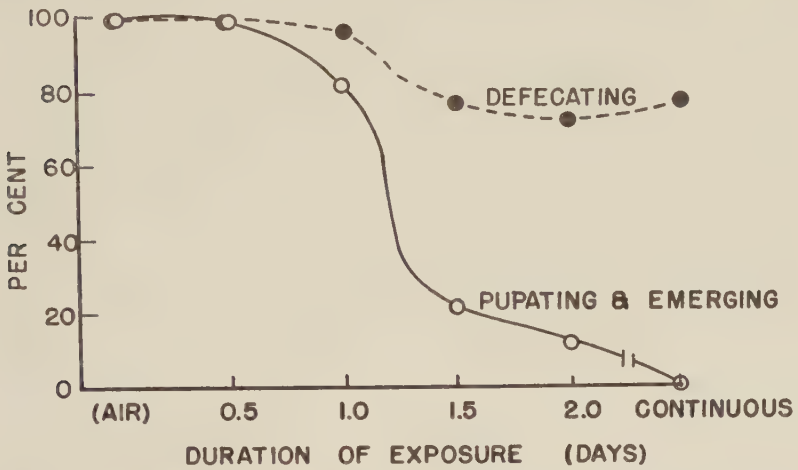

Fig. 4 The effect of varying periods of exposure to SF_6–O_2 mixtures on the development of mature larvae. The abscissa indicates the duration of exposure to SF_6. The broken line records the per cent of animals defecating; the solid line records the per cent pupating normally.

animals never emerged fully although they sometimes developed to the black developing adult stage and occasionally made unsuccessful attempts to emerge as distorted adults. However, when mature larvae were exposed to SF_6 for periods of 24 hours or less, normal pupae were formed which developed and emerged as normal adults at the same rate as air controls.

Effects on diapausing larvae. In response to specific environmental factors *Mormoniella* females produce offspring which enter diapause at the end of the last larval instar (Schneiderman, '57). About 20 such diapausing larvae were kept in a mixture of SF_6 and oxygen for 10 days. At the end

of the period they were still alive and active, apparently little worse for their ordeal.

Effects of 5 and 10 atmospheres of SF_6 on adult development. At elevated pressures SF_6 was exceedingly toxic and inhibited the further development of pupae at all stages. When development did occur it was only slow and partial and the animals never emerged.

DISCUSSION AND CONCLUSIONS

Our results show that at atmospheric pressure helium and argon when substituted for the nitrogen in the atmosphere, fail to affect the rate of development of *Tenebrio* and *Mormoniella.* We have been unable to confirm the earlier observations of Cook ('50), and cannot identify with certainty the differences in the design of our experiments which led to these contrary results. Although these gases at atmospheric pressure do not seem to affect the rate of development this does not gainsay the possibility that replacing the nitrogen in air by helium, which is only one seventh as dense, may affect certain physiological phenomena which depend upon air-density. Indeed it is well established that wing-beat frequency and flight itself are markedly influenced by helium (Chadwick and Williams, '49; Chadwick, '53). Chadwick and Williams ('49) made special note of the fact that *Drosophila* "reacted badly to . . . helium mixtures" at one atmosphere and became unresponsive to the flight stimulus (page 125). Similar responses have been reported by Sotavalta ('51) for other species maintained in helium-oxygen mixtures, and the effects were diminished when the air-density was increased by raising the helium pressure. However, aside from the special case of flight most experimental work on other forms supports the view that helium and argon are physiologically inert at atmospheric pressure, and we prefer to believe that in insects too these gases are inert at atmospheric pressure. The only circumstances under which they affect development in a different manner from nitrogen is at positive pressure.

It appears clear from the results that nitrogen and argon at elevated pressures, as well as sulfur hexafluoride at atmospheric pressure, exert narcotic effects on *Mormoniella*. Sulfur hexafluoride had the most pronounced effects and, unlike the other gases, immobilized active adults even at atmospheric pressure. Helium, on the other hand, at pressures up to 10 atmospheres had little or no deleterious effects on any stage of *Mormoniella*.

It seems likely that the principal sites of narcotic damage are the following:

1. *The proliferating cells of the imaginal discs.* Nitrogen and argon at 5 atmospheres pressure had no effect on the rate of adult development or on the ability of black developing adults to emerge, and probably no effect on developing larvae. Yet both gases considerably delayed pupation. Likewise, normal pupation was completely inhibited by continued exposure to sulfur hexafluoride for 36 hours, a treatment which permitted larval cell division and visible adult differentiation. Hence it appears that the formation of the pupa, involving as it does extensive proliferative activity of the imaginal discs, is especially sensitive to the action of the gases used in this study. Since these narcotic gases do not prevent defecation, their target is not the endocrine system of the insect which triggers off the growth process (Schneiderman, '57).

2. *Neuromuscular coordination of the fully differentiated adult.* Sulfur hexafluoride at atmospheric pressure inhibited the coordinated muscular activity of adults and of black developing adults about to emerge. Nitrogen and argon even at 10 atmospheres failed to have this effect, except insofar as it may have contributed to the failure of many pupae to emerge normally and fully after exposure.

3. *The differentiating imaginal nerve and muscle cells within the pupa and developing adult.* The proportion of pupae which failed to emerge as adults when exposed to 10 atmospheres of argon depended on the length of previous exposure: the longer this exposure, the greater the proportion failing to emerge fully. Also when the wasps were exposed

to SF_6 for a long time during their early adult development, their subsequent emergence in air was prevented. Thus it appears likely that both pupae and developing adults suffered cumulative damage from both argon and SF_6. This damage did not noticeably affect the visible differentiation of the adult, yet it greatly impaired ultimate adult emergence, suggesting that the development of coordinated neuromuscular mechanisms was being blocked. This makes it likely that the actual sites of damage were the developing nerve cells of the early pupa and developing adult, and possibly the muscle cells as well, although many more of the latter would have to be damaged to produce any discernible effect.

The results establish the order of narcotic effectiveness of the four gases on *Mormoniella* as follows: helium, no effect; nitrogen, slight effect at 10 atmospheres; argon, moderate effect at 10 atmospheres; SF_6, severe effects at atmospheric pressure. Such an order correlates well with that obtained from previous studies on vertebrates, as well as with several possibly significant chemical and physical variables, such as increasing molecular weight and increasing oil-water partition coefficient. Which properties of these gases are most relevant to their narcotic effects is a question beyond the scope of this study.

SUMMARY

Experiments were conducted to determine the effects of helium and argon on the development of the mealworm *Tenebrio molitor* and the chalcid wasp *Mormoniella vitripennis.* Helium and argon when substituted for the nitrogen in the atmosphere at any stage in the life histories, failed to affect the rates of development. On the basis of these findings we have concluded that, contrary to earlier reports, inert gases at atmospheric pressure do not accelerate the development of insects.

Experiments were also conducted to determine the effects of nitrogen, helium and argon at 5 and 10 atmospheres pressure on the development of *Mormoniella.* Helium had no effect at the pressures studied while both nitrogen and argon

retarded development. Five atmospheres of these gases delayed pupation and under certain conditions delayed adult emergence, but never delayed adult development. Ten atmospheres delayed the rate of adult development as well. The effects of argon were more severe for, while animals always emerged after exposure to 10 atmospheres of nitrogen, argon frequently prevented emergence entirely. The degree of inhibition was proportional to the duration of previous exposure.

The effects of sulfur hexafluoride on the development of *Mormoniella* were also studied. Exposing newly pupated wasps continuously to SF_6-oxygen mixtures at atmospheric pressure delayed adult development and prevented emergence. Exposing wasps to SF_6 continuously for 6 days from the beginning of adult development or for a single day at the end of adult development irreversibly inhibited adult emergence. Likewise one day of exposure to SF_6 irreversibly paralyzed active adults. However, exposing wasps to SF_6 for one day at any stage of development except the day prior to ecdysis failed to prevent emergence.

Sulfur hexafluoride often permitted embryonic development and hatching of *Mormoniella* eggs, but it completely prevented the growth and ultimate defecation of the resulting larvae.

Mature larvae exposed to sulfur hexafluoride usually defecated, but when exposed for a long enough time did not pupate normally. However, with periods of exposure of 24 hours or less normal pupae formed and these developed into normal adults.

The viability of diapausing larvae was not visibly affected by 10 days of exposure to SF_6.

At elevated pressures sulfur hexafluoride almost completely inhibited adult development.

In the Discussion, the possible sites of damage by the gases under study was considered.

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SWIMBLADDER VOLUME, BUOYANCY, AND BEHAVIOR IN THE PINFISH, LAGODON RHOMBOIDES (LINN.)

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TWO FIGURES

The evolutionary origin of all land vertebrates is traceable to the aquatic environment of primitive seas. The apparent disarray of structural variations among animals when viewed species by species gives way to underlying sequential order, when compared structure by structure. Unfolding patterns are displayed along phylogenetic pathways which lead from teleostean fish to modern birds and mammals. As continuity among structural variations becomes matched by evidence for corresponding functional modes, and these, in turn, find concordance with advancing behavioral and environmental knowledge, the transcendent theme of biological affinity among all vertebrates becomes more apparent.

The trend of structural modification in animals can be correlated with two basic physical considerations: First, the relative *magnitude* of force which the animal itself can develop in correspondence to each force in the environment; and, secondly, the *rate* at which each force in the animal can be developed. For physiology, the first stresses such ubiquitous mass influences as gravitational, thermal, and compressional forces; the second stresses such particulate characteristics as the translational rates and diffusion rates of essential metabolic materials. Since these elements are of uneven distribution among the various environments, a third and uniquely biological specialization lays structural trends; namely, the de-

gree of homeostatic regulation that evolves to select the time and place, as well as magnitude and rate, at which the separate forces in the animal's economy will operate.

A biologically efficient animal is highly adaptable to a given environmental force. Comparative biology takes special interest in those features among animals which confer unusual adaptability and independence: Features which provide a large range through space; a high rate of energy exchange; extreme sensitivity and selectivity for variations in environmental forces; large capacity for retention, correlation, and integration of environmental energy samples; and extreme selectivity and precision of energy output. These characteristics are especially expressed in locomotion, respiration, sensory reception, and behavior. They help to account for the interest which the teleostean swimbladder has long held for animal physiologists.

Comparative anatomists have considered the swimbladder to be the origin of the vertebrate lung (Romer, '50). In some modern fish, like those of the Paraguayan Chaco swamps (Carter and Beadle, '31), it has been shown to acquire function as a supplementary respiratory organ or even, in some species, to become an essential lung. The latter fish die in oxygen-deficient water if denied access to air, even though they may also have functional gills (Carter, '57). Thus a pattern of modification which could lead to the amphibian lung is illustrated. However, there is evidence from paleontology that the swimbladder of modern fish and the lung of amphibians may have their origin in the respiratory bladder of primitive teleosts (Romer, '57). Whatever the primitive relationship, the close affinity of these structures is clear.

A dominant feature of the aquatic environment is that large change in pressure accompanies small change in vertical position. With the exception of gravity, which can only signal rate and direction of movement, and orientation in space with reference to intrinsic axes of symmetry; and light, which only signals at moderate depths and a portion of the time; a far ranging, fast moving fish finds no other variable for spatial

orientation. There is no solar or celestial horizon, no large asymmetrical contact pressure, no echo differentiation, no large, uniform chemical or thermal gradient; none of the common energy variables which may play on shallow-water and terrestrial animals. Existence of a pressure receptor and response mechanism among fish seems highly probable — as probable, in fact, as a vestibular apparatus. Furthermore, an appropriately sensitive mechanism would seem to demand a responsive gas volume, because of the low compressibility of liquids.

It is not surprising, therefore, that the teleostean swimbladder has long been considered (e.g., Baglioni, '08) a pressure receptor organ. Jones and Marshall ('53) have extensively and critically reviewed the literature on the swimbladder. It is surprising that they find this function to be, even now, highly uncertain, on the basis of experimental evidence. They cite one experiment which has produced important supporting evidence: Vassilenko and Livanov ('36) found that action potentials in the vagus nerve of carp varied with swimbladder pressure.

Emphasis in many studies of swimbladder function has centered on its now well established role as a hydrostatic organ and on processes by which its volume is adjusted to changes in ambient pressure. In species with a duct to the pharynx, air may be swallowed or released for volume control. These physostomes may also secrete gas; and all physoclists, lacking the duct, secrete and absorb gas from the bladder. Direct analyses of bladder gas have shown secretion of oxygen, carbon dioxide, and nitrogen against prevailing pressure, even beyond 250 atmospheres, in a large variety of fish (e.g., Scholander and Van Dam, '54). Resorption of gas is by way of a special structure, the oval, for the bladder is generally impermeable. These processes for volume adjustment, though large in magnitude, are shown to be slow (e.g., Jones, '51), a factor which severely limits the rate of vertical migration.

The body of a fish is generally denser than the environment. Fat is one component, aside from gas, which has appreciably

lower density than water. Thus fat deposits can to some extent offset the tendency to sink and diminish the locomotor activity needed to remain at any level. But complete buoyancy adjustment to any depth is most readily and completely accomplished through a critically regulated gas volume; such is the swimbladder, and a majority of teleosts have one. Even the presence of a gas bladder for sensory function would necessitate such critical regulation because of its buoyancy influence. The rate at which pressure within the bladder can be equilibrated with external pressure limits the range and rapidity of vertical movement, and the rate at which the volume of the bladder can equilibrate the total density of the fish at any level determines the rapidity with which neutral buoyancy and poised immobility is achieved. From such considerations, a reflex muscular control of swimbladder volume has been postulated and a number of pertinent experiments have been performed (Jones and Marshall, '53). The preponderance of existing evidence seems to deny any muscular control in physoclists and ascribes to secretion and absorption the complete regulation of volume (e.g., Jacobs, '32).

One common method to study swimbladder function is to place fish in a convenient vessel and apply pressure to simulate various depths, while observing their orientation behavior (e.g., Brown, '39). Under similar conditions, bladder gas has been sampled and analyzed (Rostorfer, '42); or bladder gas has been removed or air added and subsequent orientation observed (e.g., Remotti, '24). Behavior has also been studied when eyes and utriculi (Von Frisch, '34), and fins (Brown, '39) are removed. But no studies have been found in which the volume and pressure of the swimbladder were directly and continuously measured and controlled while the free behavior of the fish in relation to depth was observed. Such experiments are the subject of this report.

MATERIALS AND METHODS

The pinfish, *Lagodon rhomboides* (Linn.), is a physoclistous marine teleost, Order *Percomorphida*. It is common in coastal

waters of North Carolina, around the Duke Marine Laboratory, until late in November. At maximum growth, it is about 25 cm long. Its swimbladder (fig. 1) conforms dorsally and laterally to the body cavity, to which it adheres, its ventral surface forming a membrane over the viscera. Examination of this swimbladder revealed an unusual feature: a pair of long, slender dorso-lateral lobes connected by ducts to the anterior end and embedded in muscle along each side of the vertebral column. The swimbladder itself is centered at the intersection of the longitudinal and dorso-ventral axes of symmetry of the fish;

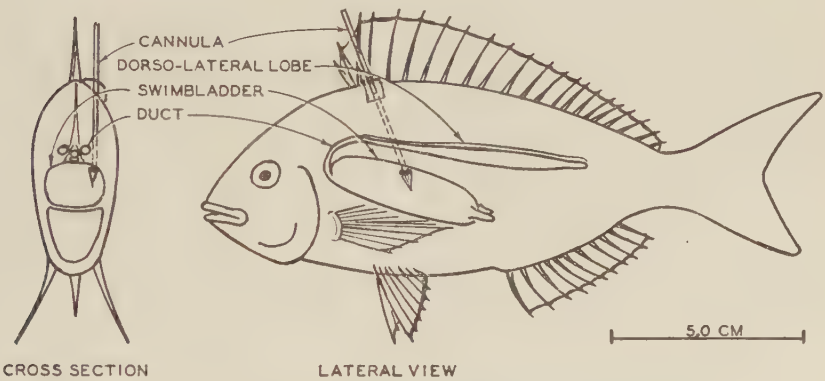

Fig. 1 Longitudinal and cross-sectional diagrams of pinfish to show structure and orientation of swimbladder and position of cannula.

it slopes downward from the anterior end at an angle of about 20 degrees with the longitudinal axis. It has a gas gland occupying about one-half of the anterior floor, and there are two short, mid-lateral projections at the posterior end. The particular orientation of the bladder and its dorso-lateral lobes are noteworthy in relation to passive responses of the fish which will be described.

An indwelling cannula, connected to a hypodermic syringe and manometer, provided for control and measurement of swimbladder changes in the free-swimming fish (fig. 2). Fine polyethylene tubing (size PE50, I.D. 0.023″ × O.D. 0.038″) was used to connect the cannula in the bladder, through a

swivel, to a 1.0 mm bore manometer and a syringe. This tubing allowed normal orientation and free movement of the fish in the tank. Since direct observation of behavior was necessary, direct pressure readings were made concurrently, rather than recording. The tank had a capacity of 20.0 liters; and

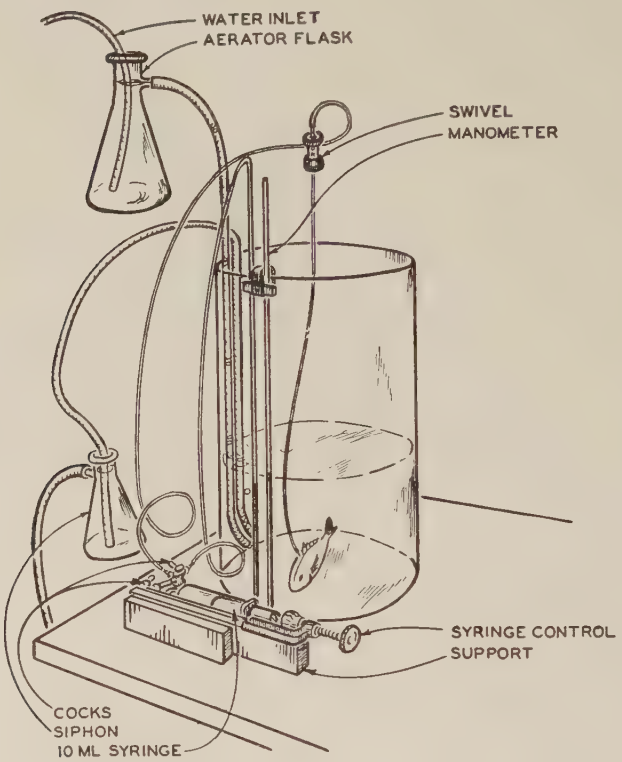

Fig. 2 Apparatus for measurement of pressure and control of volume in the swimbladder of free-swimming pinfish.

incoming sea-water was aerated, and its level in the tank was established by the position of a constant-level syphon. The total dead space of the system from cannula to syringe was 2.5 ml, including manometer and valves.

The cannula was made from 23.0 gauge hypodermic needle stock. Its point was tapered about 35° and merged into a tapering hub which was built up of plastic cement. The hub

extended back from the point about 2.0 mm, where it reached a diameter about three times the needle stock; here a flat shoulder was formed. When the cannula was passed into the swimbladder, this shoulder restricted its withdrawal with movements of the fish. The softness of fish muscle makes the hub essential to secure the cannula. A rectangle of rubber about 5.0 mm by 7.0 mm was cut from the skirt of a serum bottle cap, and the other end of the cannula was pushed through a central puncture so the naturally concave surface of the piece was toward the point. This formed a collar which could be adjusted, after the cannula was inserted in the swimbladder, to bear against the dorsal fin and restrict rotation of the cannula as the fish turned. The position of the cannula on insertion is shown in figure 1. To avoid the sinus-like branches of the dorsal aorta, the lateral lobes, or termination in muscle, required about 5 attempts at insertion for each successful preparation. The best placement slid the tip of the cannula under a rib so the hub caught on it and resisted withdrawal into muscle. Such a preparation was free in the tank for as long as 5 days, unattended for periods of two to 8 hours, without disturbance of the cannula.

The swivel is essential to the free movement of the fish, because the polyethylene tubing and cannula will not accommodate circling. The swivel was made from a piece of 8.0 mm glass tubing 2.0 cm long. A small serum bottle stopper was inserted into the bottom, a 15.0 mm piece of the same needle stock which was used for the cannula was in turn inserted through the stopper, a short piece of polyethylene tubing was fitted around the inside portion of the tube thus formed and pushed down to the stopper as a retaining collar, and a drop of glycerin for lubricant and a drop of mercury for seal was placed within, around the needle stock. The bottom tube was rotated until it worked freely in the stopper, and the polyethylene tubing from fish and three-way cock was connected to appropriate ends of the swivel. The swivel did not leak with pressure up to 180.0 mm Hg; a much higher pressure than could be retained at the cannula.

Fish were anesthetized, to facilitate insertion and location of the cannula, by placing them in an aquarium of urethane solution (1.0% in seawater). The solution was continuously aerated, and the urethane effect abolished reflex response to handling in 5–10 minutes.

Mercury was used in the manometer for some measurements, and water for others. For purposes of tabulation and because the ambient pressure variable was depth of sea-water, mercury readings are converted to sea-water equivalents by use of two standard density values: 1.029 for sea-water of 3.5% salinity at 20.0°C, and 13.54 for mercury. Variations of sea-water at the Duke Station in both salinity and temperature are known to range considerably from the selected factors, but the salinity was not followed in these studies. These variations are not significant within the scope of pressure measurements and effects.

The volume of gas in swimbladders and lateral lobes was measured by immersing the fish, or ligated, excised lobe, in a vessel of acidulated water; opening the previously exposed bladder, or the lobe, with a probe; collecting the displaced gas by displacement of water in an inverted funnel closed with a serum bottle cap; transferring gas by syringe and measuring in a gasometric pipette (McCutcheon, '43). The fish were usually acclimatized at a depth of 18.5 cm of water before measurement, and the measured volumes were reduced to physiological conditions of temperature (20.0°C) and water vapor (saturated), and to standard sea-level pressure (760.0 mm Hg). Total volume of fish was measured by volumetric displacement through total immersion of the fish.

Since the passive and overt behavioral responses constitute a primary element of this study, a protocol method of reporting these observations is used as the most informative and economical. This required selection of appropriate time intervals to display the reproducible, classifiable behavior patterns. The selection was made from 108 hours of observation on 5 preparations, none of which failed from disturbance of the cannula or other reasons in less than 24 hours.

None of these five fish was under observation less than 7 hours, and the longest observational sequence was 49 hours in 5 days. These and other specimens were also observed while they were free in the stock aquaria for verification of certain experimental results.

Stock pinfish were easily kept in tanks when supplied with flowing sea-water and fed any kind of fish scrap; some fish that were used for bladder volume determinations after three months in these conditions had grown larger. Pinfish could not be taken by hook or net inside the Beaufort Inlet after November 15, where they had been plentiful before that time. They apparently had migrated, as a result of falling water temperature, seaward to the warmer gulf stream area. This was not anticipated, and the table of swimbladder volumes is curtailed for lack of time to extend fishing activities.

RESULTS

Orientation and acclimatization of the pinfish, under these experimental conditions, is largely under operative control. It is directly correlated with the volume of the swimbladder, it is predictable in terms of the pressure differential between bladder and depth, and it is quite stereotyped. For convenience of analysis and tabulation, the following behavioral elements and abbreviations are established:

a. *Positional fixation.* When disturbed or displaced, following a stationary period of about five minutes, a fish returned regularly to the exact location in the tank from which it started. Return followed swimming caused by any mild disturbance, such as movements of observer, or mechanical displacement. Such localization persisted after full buoyancy compensation for as long as 12 hours.

b. *Immobilization.* A fish placed in neutral or negative buoyancy, its swimbladder pressure (SBP) within about + 3.0 cm H_2O of ambient pressure, lay for periods of 20–60 minutes at a time with ventral surface on bottom, fins relaxed and folded, with no movement except breathing (see protocols). Reduction to or below this level of swimbladder volume

PROTOCOL 1

Adjustment and acclimatization to positive buoyancy

Fish no. 10306 — St'd. length 14.3 cm; dorsum to SB center 2.5 cm. LA° approximated, and depth at SB center when tilted is estimated. Pectoral beat amplitude and frequency estimated on unit scale 0–5; see Protocols 2 and 3 for measured rates; negative sign on frequency indicates occasional or intermittent.

TIME	SWIMBLADDER		FISH				Behavior — Remarks
			Position		Pectoral		
	Vol.[1]	Pressure	Depth [2]	LA°	Ampl.	Freq.	
	ml	*cm H_2O*	*Cm*				
11: 15	0.0	34.21	34.8	0	0	0	IS. On bottom
11: 20	0.4	35.25	34.8	0	0	0	IS. On bottom
11: 20	0.6	36.05	34.8	0	0	0	IS. On bottom
11: 20	0.8	36.84	34.8	0	0	0	IS. On bottom
11: 20	1.0	37.62	34.8	0	0	0	IS. On bottom
11: 20	1.4	38.15	34.8	0	1	1 —	PB slight. Drifts up at head. Pectorals restore.
11: 20	1.8	38.16	34.8	0	3	3	Quivers, then cruise up 3.0 cm depth and back.
11: 25	2.0	40.79	34.0	— 20	5	5 +	Excited; fins extended; tail thrust; one cruise.
11: 27	2.0	38.15	34.0	— 20	5	5	One cruise to 3.0 cm depth and back.
11: 30	2.4	41.05	33.8	— 20	5	5	Cruise to 5.0, then 12.0 cm depth and back.
11: 48	0.0	40.51		— 10	4	4	Tail thrust contin., vent. fins touch bottom.
11: 50	0.0	40.79		— 5	4	4	As above.
11: 51	0.0	42.89		— 5	4	4	As above.
11: 52	0.0	40.51		— 5	4	4	Press. reduction followed one cruise to 12.0 cm.
11: 55	0.0	39.48		— 5	3	3	As 11: 48–11: 52.
11: 56	0.0	40.51		— 3	3	3	Vent. fins touching bottom.
11: 57	0.0	39.48		— 3	3	2	As 11: 52.
11: 58	0.0	38.95	34.5	0	3	2	
12: 00	0.0	39.48	34.5	0	3	2	
12: 05	0.0	38.15	34.5	0	2	2	After twice up to 12.0 cm and return.
12: 45							Repeated 11: 52 to 12: 05 sequence, 2/min swim up to about 12.0 cm, circle once, and return bottom location and hold by fin movements.
12: 47	0.0	38.72	33.8	10	0	0	Fins extended but still.
1: 27	0.0	39.48	33.8	5	0	0	As above.
1: 28	0.0	35.80	15.0	0	2	2	While at 15.0 cm for 30.0 secs on one cruise.
1: 29	0.0	40.79	33.8	0	0	0	On reaching bottom location.
1: 29	0.0	37.62	34.5	0	0	0	While "quivering" and nestling into bottom position.
1: 35	0.0	37.62	34.5	0	0	0	At rest, fins slightly extended, ventrals on bottom.
1: 40	0.0	38.15	34.7	0	0	0	IS. On bottom. Absorbed 1.0 ml/130 min.

[1] Total volume added since initial reading.

[2] At center of SB.

(SBV) from any level of positive buoyancy and compensatory activity, or mechanical displacement upward, or movement from strong visual disturbance, was followed by immediate resumption of this *immobilized state* (IS). Responsiveness to visual or vibrational stimulation was greatly reduced, as though "activating" centers were largely depressed.

c. *Longitudinal axis trim.* The central longitudinal axis (LA) of the fish was subject to angular displacement of the head; that is, horizontal tilt; by appropriate fin movement or by alteration of SBV (protocols 1, 2, 3). From neutral buoyancy, *gradual* increases of SBV less than about 10.0% (0.3 ml/3.0 ml) caused a proportionate passive drift upward of the head to a limit of about $+15°$. At any angle above $+5°$ the fish might respond, depending on rate of increase, and above $+15°$ it invariably responded by compensatory pectoral beats to restore and hold horizontal position. The limits of these angles were not critically established.

d. *Vertical axis trim.* The central dorso-ventral axis (DA) of the fish was subject to angular displacement from vertical; that is, lateral tilt; by appropriate fin movement and, more remarkably, as a result of asymmetrical visual disturbance alone, with fins at rest. For example, when the fish was in neutral buoyancy (IS) and the observer's hands were resting prone on the table beside the tank, parallel to the fish, elevation of the index finger alone of one hand would cause the fish to lean in that direction (protocol 3). It would tilt sidewise to about 20° in about 0.3 sec, and return to vertical in about 2.0 sec. The degree and maintenance of displacement was roughly proportional to the extent and duration of disturbance. Accommodation of this response occurred but was not critically analyzed.

e. *Local pectoral compensation.* With imposed positive buoyancy (PB) from increased SBV exceeding about 10%; that is, outside limits stated in (c) above, as well as with strong negative buoyancy (NB); appropriate compensatory movements of pectoral fins occur. In PB the pectoral, ventral, and dorsal fins erect somewhat proportionally to the degree of

PROTOCOL 2

Adjustment and acclimatization to positive and negative buoyancy

Fish no. 11136 — St'd. length 14.7 cm; dorsum to SB center 3.2 cm. LA° approximated, and depth at SB estimated during tilt. Pectoral beat amplitude and frequency estimated on unit scale 0–5 except where counts are noted; negative sign on frequency indicates occasional or intermittent; see protocol 3 for additional rates.

TIME	SWIMBLADDER		FISH				Behavior — Remarks
			Position		Pectoral		
	Vol.[1]	Pressure	Depth [2]	LA°	Ampl.	Freq.	
	ml	*cm H_2O*	*Cm*				
1: 30	0.0	34.21	29.6	— 5	4	71/m.	Excited; dorsal fin erect. PB.
1: 35	+ 0.1		28.6	— 8	5	72	As above. Total depth of water 38.4 cm.
1: 38	— 0.3		23.2	0	3	48	As above. Dorsum to SB center 3.2 cm.
1: 42	— 0.2		25.1	0	0	0	IS.; dorsal fin down, fins folded.
1: 43	+ 0.1	26.84	23.6	8	0	0	Slight PB, floats up very slowly at head.
1: 46	— 0.1	37.10	35.1	0	0	0	IS.; sinks to bottom.
1: 47	+ 0.1	26.83	23.5	8	0	0	As 1: 43.
1: 48	+ 0.1	34.21	30.6	0	3	32	Dorsal fin erect; ventrals extend slightly.
1: 55	0.0	33.15	27.6	0	0	0	Poised this level for 30–45 sec.; then slow pectoral beat moves down 2–4 cm.
1: 57	0.0	29.74	25.6	5	0	0	Slow drift up, poise, then down as above.
2: 08	0.0	36.84	34.1	0	2	30	As above, longer intervals.
2: 21	0.0	36.58	33.6	0	1	1 —	Dorsal relaxed, other fins extend slightly.
2: 24	— 0.05	38.15	35.1	0	0	0	IS. Absorbed 0.15 ml/38 min.
2: 30	+ 0.15	34.21	31.6	— 5	3	56	Pectorals hold in position.
2: 34	0.0	31.58	29.6	0	1	1 —	Dorsal fin relaxed. Compare 1: 30 above.
2: 38	+ 0.4	34.49	30.6	— 20	5	88	Excited; tail thrust; dorsal erect.
3: 00	0.0	34.21	31.6	— 10	5	72	Holds one position; drifted to 19.0 cm twice.
4: 20	0.0	36.84	35.1	2	0	0	Slight PB.
4: 20	— 0.1		35.1	0	0	0	IS.
4: 20	— 0.3		35.1	0	0	0	IS.
5: 25	+ 0.3		34.0	8	1 —	1 —	Slight PB.
5: 25	— 0.1	36.84	35.1	0	0	0	IS.; gain approx. 0.1 ml since 4: 20.

[1] Total volume added (+) or removed (—) since previous reading.

[2] At center of SB.

buoyancy. But pectorals alone will beat, if these can hold the head down near resting position. As tail rises with increasing buoyancy, however, increased amplitude and frequency of pectoral beats develops, until a LA angle of about — 20° is reached (protocols 2, 3).

f. *Locomotor compensation.* As increasing PB imposes more than —20° LA angle, supplementary tail thrusts of increasing magnitude and frequency develop. At high buoyancy, about 30% increase SBV (1.0 ml/3.0 ml) and above, occasional to frequent cruises and rushes around tank intervene; these follow a regular pattern. At limits of buoyancy which fin compensation can withstand, the fish holds its mouth at the bottom resting position and swims with all fins strongly erect, with maximum tail-sculling and pectoral beat; thus it may hold LA within 25° of vertical for intervals as long as 10 minutes. This activity is described as "boring" (protocol 3). Boring may be accompanied by sweeping movements of the anterior end in 3.0–6.0 cm arcs. Boring is interrupted at regular intervals (3 to 10 min.) by one or two cruises around the tank as much as one-third from the top, and return (protocol 1). The compensation pattern of increasing buoyancy is reversed as SBV decreases, by absorption of gas (or removal by syringe), until the fish can finally remain IS with LA horizontal, ventral fins touching bottom slightly extended, other fins folded. Van Bergeijk ('54) describes a locomotor reflex of Xenopus tadpoles, in response to hydrostatic pressure changes, with orientation behavior like the pinfish.

With imposed NB from decreased SBV of more than about 10% (0.3 ml/3.0 ml) from neutral, pectoral fins also fold and the fish rests (IS) on ventral surface, DA vertical, for periods of three to 10 minutes. Then pectoral beats will develop in keeping with the degree of negative buoyancy. The thrusts of pectorals are opposite to those during PB and appropriate to hold the fish off bottom except for ventral fins in contact. These movements will persist for 10–30 minutes; unless the fish is disturbed, even mildly, when they cease at once. Pectoral beats will resume as disturbance effects subside, and continue

PROTOCOL 3

Adjustment to increased water level and positive buoyancy

Fish no. 11176 — St'd. length 13.1 cm; dorsum to SB center 2.9 cm; SBV 3.36 ml. LA° approximated, and depth at SB center estimated during tilt. Pectoral beat amplitude estimated on unit scale 0–5; see protocol 2 for additional rates.

TIME	SWIMBLADDER		FISH				Behavior — Remarks
			Position		Pectoral		
	Vol.[1]	Pressure	Depth [2]	LA°	Ampl.	Freq.	
	ml	*cm H_2O*	*Cm*				
8: 37	0.0	14.50	11.4		0	0/m.	Fins folded; ventrals on bottom; IS.
8: 50	— 0.4	16.50	13.2		1	102	As above except slight pectoral beat. NB.
8: 55	0.0	16.70	13.6		1	102	SBP increases steadily.
9: 00	0.0	17.20	14.0		1	103	
9: 10	0.0	17.30	14.5				
9: 15	0.0	19.50	16.1				
9: 20	0.0	20.50	17.1				
9: 25	0.0	25.60	22.1				
9: 30	0.0	28.10	25.1				
9: 35	0.0	29.30–29.90	26.9				SBP rises slowly to first value, holds, then jumps to second.
9: 40	0.0	31.70–32.30	28.7				
9: 45	0.0	33.10–33.90	30.4				As above.
9: 50	0.0	35.90–37.00	31.7				As above.
10: 00	0.0	36.88	31.9		0	0	Fins folded; IS. Move hand or tap glass, fish tilts 10–20° laterally.
	+ 0.3	36.90	31.8	2	1	48	Dorsal folded, no tail thrust. Water level constant.
	+ 0.1	36.90		0	2	54	As above; pectorals hold in position on bottom.
	+ 0.2	36.70		— 5	5	66	Dorsal erect; tail thrust holds head position.
	+ 0.2	36.86			5	78	As above; cruises once around tank occasionally.
10: 12	+ 0.2	36.68			5	90	As above; cruises frequent; returns to position.
10: 16	+ 0.2	36.60			5	102	As above; cruises are about 1.0 cm off bottom.
10: 17	+ 0.2	36.90					As above.
	+ 0.4	37.10		— 35	5	114	Cruises continuously.
	+ 0.8	38.70					As above.
10: 29	+ 0.4	38.78		— 45			"Boring" at one spot on bottom.
10: 33	+ 0.6	41.02					Boring, and sweeping in 60–120° arcs.
10: 50	+ 1.0	43.10		— 55			As above, with occasional circling.
11: 14	— 4.3	35.58	31.7	0	1		Fins folded; ventrals touch bottom. NB.
11: 15	+ 0.2	35.60	31.9	0	0	0	Fins folded; ventrals on bottom; IS.

[1] Total volume added (+) or removed (—) since previous reading.

[2] At center of SB.

intermittently until secretion of gas restores neutral buoyancy (protocol 2). Alternatively, with stronger SBV decrease of from 10 to 30%, the fish will swim up in the tank using pectoral beats alone and holding LA horizontal. Appropriate pectoral beat will then hold the fish, normally oriented, just below surface or at some depth consistent with its buoyancy. If neutral buoyancy is achieved at surface or intermediate depth, fin motion may stop (IS) and fish may remain poised at that position (protocol 4). If then disturbed, it may immediately swim to bottom and hold, either IS or with pectoral beats. On reaching bottom, if IS with slight PB it might slowly float back to former level. When poised IS off the bottom and the disturbance is slight, however, the fish may respond in another way. It may, after momentary flutter of pectoral fins while SBP increases about 1.0 cm H_2O, just sink IS to bottom.

g. *Compressatory compensation.* When a pinfish has a positive pressure differential with environment by an excess SBV less than 0.5% above neutral, it can actively compress to neutral. This accompanies three kinds of behavior: (a) extend fins and quiver in location; (b) cruise up 10–15 cm, circle once or twice, and return; (c) what will later be described as "yawn." Such maneuvers coincide with an increase in bladder pressure of from 0.1 to 3.0 cm H_2O. This seems to be the upper limit of muscular control. Sensory disturbance of a fish in neutral buoyancy, such as one tap on tank or movement of hand, can also result in similar pressure increase, lasting about 20 seconds (protocol 4). An increase of 0.5 cm H_2O pressure in behavioral compensation may last 10–15 minutes. Again, the increased pressure after a maneuver may, within one minute, drop 1.5 cm below the level at start; then gradually rise to within 0.2 cm of start during 4–5 minutes, pectoral beats continuing, when maneuver is again repeated (protocols 1, 3). This stereotyped behavior may continue, with decreasing frequency, for two hours under conditions of these experiments. It would continue, in general, until absorption of gas to neutral range is complete.

PROTOCOL 4

Responses near neutral buoyancy

Fish no. 11196 — same as 11176, protocol 3. LA° approximated. Pectoral beat and amplitude estimated on unit scale 0–5; see protocols 2 and 3 for measured rates.

TIME	SWIMBLADDER		FISH				Behavior — Remarks
			Position		Pectoral		
	Vol.[1]	Pressure	Depth [2]	LA°	Ampl.	Freq.	
	ml	*cm H_2O*	*Cm*				
12: 27	0.0	5.68	3.1	0	0	0	IS; on bottom.
12: 27	0.0	7.36	3.1	0	0	0	SBP rise with light tap on tank, lasts 20 sec; IS.
12: 29	0.0	5.72	3.1	0	0	0	IS.; on bottom.
12: 29	0.0	6.08	3.1	0	1	1 —	Slight pectoral mov., then yawn and SBP rises.
12: 42	0.0	5.80	3.1	0	0	0	IS.; on bottom.
6: 56	0.0	20.66	18.1	0	0	0	IS.; on bottom. NB.
7: 40	0.0	9.84	18.1	0	2	3	Pectorals hold at 6.9 cm from surface.
7: 40	0.0	10.28	18.1	0	2	3	Fins stop; sinks to 10.9 cm; fins return to 8.9 cm.
	0.0	9.78	18.1	0	2	3	Repeats above about 1/min; SBP at 6.9 cm depth.
8: 00	0.0	12.24	18.1	0	0	0	At 10.5 cm depth during above cycles.
8: 05	0.0	5.40	18.1	0	0	0	IS.; dorsum at surface.
8: 21	0.0	20.84	18.1	0	0	0	Slight pectoral beat at surface, then sank (IS) to bottom.
8: 22	0.0	12.90 — 15.10	18.1	3	2	3	Slowly toward surface; up and down between 8.9 and 13.9 cm depth slowly; yawn causes SBP rise noted, after which fish settles 1.5 cm.
8: 55	+ 0.2	13.64	18.1	0	0	0	IS.; 11.0 cm from surface.
9: 03	+ 0.2	10.06	18.1	0	0	0	Motion of my hand caused fish swim by pectorals only from 11.0 cm (IS) down to 15.0 cm; then fins stop and fish floats back to 10.9 cm; then slowly to 8.0 cm. IS.
9: 20	— 1.6		18.1	0	3	4	Pectorals beat against NB; on bottom.
9: 21	+ 0.4	21.46	18.1	0	0	0	IS.; on bottom.
9: 22	+ 1.1	13.70	18.1	— 5	4	5	Pectorals beat against strong PB; rises in tank.
9: 24	— 0.9	21.10	18.1	0	1	1 —	Slight NB; slight pectoral beat with ventral fins on bottom.
9: 26			18.1	0	0	0	Yawn; 9: 21–9: 24 series repeated 18 times, yawn after 1–3 min.

[1] Total volume added (+) or removed (—) since previous reading.

[2] At center of SB when fish at bottom.

h. *Volume adjustment compensation.* All imposed changes from neutral buoyancy, through alteration of SBV, were initially corrected by an appropriate combination of compression, pectoral beat, and locomotor activity. These actions developed progressively as SBV departed from that of neutral buoyancy at a fixated position (protocols 1, 2). These

TABLE 1

Rate of volume adjustment by absorption-secretion (Δ denotes change)

FISH	Δ VOL.	Δ PRESS.[1]	TIME	ML/HR.
No.	*ml*	*cm H_2O*	*min*	
10267	0.38	— 1.33	95	0.240
10271	0.30	— 1.20	80	0.225
10273	0.30	— 1.31	80	0.225
10301	0.90	— 2.90	180	0.300
11132	0.15	—	38	0.237
11175	0.10	— 0.10	28	0.214
11196	1.50	—	310	0.290
			absorption av.	0.247 ± 0.034
10306	0.60	0.25	315	0.114
11135	0.10	—	65	0.092
			secretion av.	0.103

[1] Change in swimbladder pressure during period.

TABLE 2

Pinfish body and swimbladder volume measurements. Gas saturated at 20°C, 760 mm Hg

FISH	BODY			SWIMBLADDER			BLADDER/BODY
	Length		Volume	Volume			Volume
	Total	St'd.[1]	Total	Bladder	Lobes	Total	Total
No.	*cm*	*cm*	*ml*	*ml*	*ml*	*ml*	
128	11.2	9.8	—	1.70	—	—	—
1211c	12.6	10.2	30.8	0.91	0.12	1.03	0.034
1211b	14.8	12.1	46.9	—	0.26	—	—
1117	16.2	13.1	—	3.36	—	—	—
1210b	16.1	13.6	61.7	3.05	0.15	3.20	0.052
1211a	16.7	13.8	67.6	3.09	0.35	3.44	0.051
1210a	17.1	14.3	79.8	3.84	0.55	4.39	0.055
							av. 0.048

[1] Length to base of tail fin.

TABLE 3

Relationship between swimbladder volume and pressure (Δ denotes change)

FISH	WL [1]	Δ VOL.	Δ VOL. [2]	Δ PRESS.	SBP [3]	ΔV/ΔP	ΔP/ΔV
No.	*cm*	*ml*	%	*cm H_2O*	*cm H_2O*	*ml*	*cm H_2O*
10301	33.3	1.2	27	4.99	7.21	0.24	4.16
	33.3	— 2.6	— 59	— 6.30	0.91	0.41	2.42
	33.3	2.4	55	6.84	7.75	0.35	2.85
	35.0	— 2.0	— 46	— 3.43	— 0.28	0.58	1.71
11171	28.8	4.6	124	6.22	14.30	0.74	1.35
	32.1	— 4.3	— 116	— 4.50	3.48	0.95	1.05
11172	32.0	— 1.4	— 38	— 2.22	1.30	0.63	1.59
						av. 0.55	av. 2.16

[1] Water level from surface to center of swimbladder.

[2] Neutral buoyancy volume estimated from table 2.

[3] Difference between swimbladder pressure and water level.

activities also progressively subsided as gas absorption or secretion reestablished neutral buoyancy.

Data for rates of absorption and secretion are in table 1. Those for secretion include only two intervals, because compensation for negative buoyancy was much slower, and it appeared to be inhibited for long periods by contact with bottom IS. It was not, therefore, under observation as often as positive buoyancy.

TABLE 4

Swimbladder pressure at hydrostatic equilibrium (neutral buoyancy)

FISH	WATER LEVEL [1]	SBP	SBP-WL
No.	*cm*	*cm H_2O*	*cm H_2O*
10258	37.40	41.05	3.65
10251	31.50	35.24	3.74
10261	25.75	27.89	2.14
10263	25.75	29.72	3.97
10302	33.30	37.62	4.32
11186	18.50	21.80	3.30
11199	18.69	22.60	3.91
			av. 3.59 ± 0.71

[1] From surface to center of swimbladder.

Protocols 1–4 are selected portions of three series which illustrate the various conditions of SBV, SBP, and compensation related to ambient pressure at the center of the swimbladder. These protocols are typical of data on 108 hours of continuous observation on responses to swimbladder changes of varying degrees from neutral buoyancy, involving 5 preparations. One of these fish was kept for 120 hours without difficulty, by placing it in strong negative buoyancy when it was left for long intervals. It could thus be held quiet overnight.

A summary of volume measurements is given in table 2. The relationship of total SBV to body volume for the three larger fish averages 5.19%. There are insufficient data for further correlations.

A summary of SBP changes, in relation to ambient pressure, with SBV changes is given in table 3; and resting SBP at neutral buoyancy is found in table 4.

DISCUSSION

It is evident from these observations that the swimbladder makes the pinfish responsive to small pressure variations in the environment. As shown on protocol 2, the buoyancy and behavior is sensitive to 0.05 ml of gas. The change in ambient pressure necessary to produce this volume change, with an initial SBV of 3.4 ml (table 2), is $760 - \frac{760 \times 3.40}{3.45} = 11.0$ mm Hg or 14.5 cm H_2O. Thus, a fish in buoyancy equilibrium on the bottom would become active when the tide dropped or rose this amount. Protocol 3 shows such an effect with 20.0 cm rise in water level. Dijkgraaf ('42) could train trout to respond to 10–15 cm water pressure. It appears that the pinfish could range through a depth of about 30.0 cm H_2O without fin movement. Jones ('52) found about 30% external pressure variation the extreme beyond which not even fin movement could compensate, in freshwater perch. These limits for the pinfish could not be defined, because gas escaped around the cannula above 20.0 cm H_2O SBP.

The protocols show that the pinfish can compress the swimbladder gas. The compression is small, and slow drift of pressure following a rapid compression suggests a muscle tonus response. The limit of this compression was about 3.0 cm H_2O or, from Boyle's Law, 0.3% of neutral buoyancy volume. If neutral were 3.4 ml (table 2), and including instrumental dead space of 2.5 ml, this adjustment can compensate to the equivalent of about 0.02 ml of absorption, or about 5.0 min of absorption (table 1). Fish just in hydrostatic equilibrium and stationary on the bottom without fin movement (IS) were observed to begin compensatory pectoral movements, without buoyancy drift, when compression tonus varied by only 1.0–2.0 cm H_2O (e.g., protocol 1).

Calculation of volume change from pressure readings in the swimbladder is misleading, however; for internal pressure

increase is not proportional to volume change when the fish is near neutral buoyancy (table 3), and the importance of muscular control as compared to absorption-secretion may be underestimated. Table 3 indicates that a 3.0 cm H_2O change in pressure between — 0.28 and 14.30 is the equivalent of approximately 1.6 ml change in volume (3.0×0.55). Table 1 indicates that about 6.5 hours of absorption or 15.0 hours of secretion would be required to equal the muscular effect on pressure. Tension receptors, rather than pressure per se, may mediate these activities. The important factor to recognize is that near neutral buoyancy the bladder can accommodate as much as 100% volume change with only about 4.5 cm H_2O change in pressure. This is the counterpart of what is called *compliance* in pulmonary physiology. The effect of muscular tonus on the bladder is to restore rapidly over a small range the positive pressure level (+ 3.6 cm H_2O, table 4) which characterizes the poised or resting fish in neutral buoyancy (IS). It is a rapid neuro-muscular control superimposed on secretion-absorption.

The vertical axis trim response is evidence for internal control of the bladder. It may result from asymmetrical contraction of body musculature investing the lateral projections, or contraction of the wall itself. The extreme buoyancy sensitivity of a fish in hydrostatic equilibrium would allow a very small asymmetrical volume difference to produce tilt on this axis. There is no other apparent mechanism for this response, and it is not known if it occurs in fish which lack such projections. The author has found no such projections in a number of common marine or freshwater species and no reference to them in the literature. Most species, lacking them, could so respond only by asymmetrical fin activity.

Horizontal axis trim had a passive component related to the morphological slope of the swimbladder. With a fish in neutral buoyancy (IS), slow and slight increase in the volume of gas always resulted in an upward drift of the head first. Through an angle of about 15° the fish might not compensate by fin movements but remain passively displaced for many minutes.

The rate of volume increase, and therefore the rate of angular displacement as well as the total angle, determined whether the fish would compensate by pectoral fin movement and compression to return to bottom and horizontal. A rapid rise of the head through 5–10 degrees, or a slow drift which exceeded about 15 degrees, brought immediate pectoral movement to gain original position. This would be repeated, slow drifting up and rapid winnowing down, until compression and absorption restored neutral resting orientation (IS). Sometimes a yawn (e.g., protocol 4) would intervene while these compensating movements occurred; and often the fish, when it reached horizontal position on bottom, would quiver its fins and appear to nestle into position. The orientation of the bladder determines that the head must rise first when buoyancy increases.

In the sea-horse *Hippocampus brevirostris,* there is active control to distribute gas between an anterior and posterior chamber of the swimbladder; buoyancy distribution is thus compensated for horizontal swimming or resting in head-up position (Peters, '51).

The behavior of a pinfish when compensating for small volume changes in its swimbladder displayed one feature of interest to the phylogeny of neuronal patterns. This was the experimentally reproducible "yawn" (protocol 4). It is so designated because of its overt similarity to the yawn of man, dog, cat and other animals. A yawn consisted of marked extension of all fins, with dorsal fin fully erect; wide open mouth with opercula partially open and breathing stopped; and rigid extension of body and tail. These persisted about 3.0 seconds; when the fins would again fold, the mouth would close, then quickly and repeatedly open slightly and close with "smacking" movements, accompanied by similar movements of the opercula; these movements lasting another 2.0 seconds. Then the fish would resume breathing and fin movements characteristic of its buoyancy state. Yawns were seen when the fish was beating with its pectorals against slight negative buoyancy on the bottom, or when it was circling the tank in a stereotyped pattern at other levels as a result of induced negative buoy-

ancy. They also occurred when a fish was near neutral in recovery from positive buoyancy. They could be induced experimentally at any ambient pressure level (protocol 4) by first producing strong positive buoyancy for about two minutes (increase SBV), then slight negative buoyancy (reduce SBV), when a yawn would occur in 1–3 minutes.

The yawn and sigh are only occasional features of mammalian respiration, therefore their cause and significance have received little attention. The lung and swimbladder, as homologous structures with common phyletic origin, probably have homologous neuronal response and control mechanisms. It has been suggested (McCutcheon, '51) that sighs are the result of decreased tension in the walls of pulmonary units. Since the counterpart of the mammalian yawn can be induced in the pinfish by suitable manipulation of swimbladder distention, similar alterations of pulmonary wall tension may be the cause of yawns in air-breathing vertebrates.

The relationship between swimbladder volume and body volume in the pinfish (table 2) is consistent with its function as a hydrostatic organ. Measurements of the density of a variety of fish in relation to the density of their environment indicate that a swimbladder should be about 7% of the volume of a freshwater fish and 5% of a marine fish (Jones and Marshall, '53).

The small degree of muscular control which a pinfish exercises over swimbladder volume, and buoyancy, make this factor appear unimportant when compared in magnitude to secretion and absorption of gases. The latter processes give acclimatization to ambient pressure changes which may result from extensive vertical movement of the fish, large tidal or other changes in depth, or barometric pressure changes. The rates of secretion and absorption (table 1) are too slow, however, to give adjustment for rapid pressure changes. If the fish is to poise under these conditions it must use fin movements when ambient pressure change is greater than about 15 cm of water. But with pressure fluctuations less than this, the muscular SBV control can be of great importance; most

particularly when, at rest or escaping predators, fish must remain poised in a neutral background or resting on the bottom.

The usual response of a fish to disturbance from visual, vibratory, or sound stimuli was to seek contact with the bottom. These responses depended on its buoyancy and behavior at time of stimulation. If the fish was active in strong PB, it swam to bottom position and held there by fin movement. If the fish was active in NB, poised up in the tank by fin movements, the fins might stop and the fish just sink to the bottom; or the fish might swim rapidly to the bottom where the fins would fold closely to the body. With the exception of slight breathing movements the fish would appear dead, though normally oriented, and respond as an inanimate body to small water currents. Oriented eye movements followed a moving object; passive lateral tilt could be induced as previously described; otherwise the fish was markedly unresponsive. A fish was invariably put into this state (IS) by a suddenly induced NB through rapid withdrawal of gas, whatever its existing buoyancy and position in the tank. Left undisturbed in such negative buoyancy, it would eventually begin appropriate movements of the pectorals to hold it in position with the belly about 0.1 mm off the bottom, but with ventral fins and tail in contact. With any disturbance, these movements would cease and the fish would come heavily to rest again.

Copeland, ('52) has suggested, from an interesting experiment with *Fundulus,* that receptors for a swimbladder acclimatization reflex may exist in muscles of the pectorals. With the fish in flowing water, he demonstrated secretory acclimatization of bladder volume to dynamic positional equilibrium. The amount of gas significantly exceeded specific gravity requirements at that depth. Brown, ('39) found that swimbladder acclimatization proceeded when the pectorals of guppies were removed, but this would not have eliminated the muscular reflex. The question remains whether this muscular stimulus is essential to the secretory reflex. The amount of pectoral movement in pinfish which accompanied buoyancy

compensation by gas exchange seemed too limited in many series to be wholly responsible. Specific experiments are needed to provide an answer. Protocols 2 and 3 are consistent with the observations of Jones ('52) on freshwater perch, that pectoral beat is proportional to change of swimbladder volume over the range of buoyancy variation that behavioral compensation is effective.

When a pinfish achieves hydrostatic equilibrium through secretion or absorption, or when it was placed into such equilibrium by manipulation of the syringe, the bladder pressure is about 3.60 cm H_2O above ambient pressure. From this level down to ambient pressure is also the range through which bladder pressure changes with certain non-motile activities of the fish; activities which do not cause positional changes, but which do alter bladder volume in a direction which is consistent with the buoyancy requirements of the fish. Such changes appear to be reflex muscular effects with an important role in buoyancy regulation. These observations are contrary to the conclusions of Jacobs ('32) who found no evidence of any such volume regulation in the freshwater perch.

SUMMARY

Orientation and behavior of a pinfish in response to changes in swimbladder volume could be classified into 8 stereotyped patterns: (a) positional fixation, (b) immobilization, (c) longitudinal trim, (d) vertical trim, (e) compressatory compensation, (f) local pectoral compensation, (g) locomotor compensation, (h) volume adjustment.

The buoyancy of a pinfish is affected by slight volume changes in its swimbladder which may not directly stimulate a pressure sensitive reflex. Slight changes will produce angular displacement, through upward drift of the head, to affect compensating vestibular and visual reflexes. The fish also appears to respond by a direct swimbladder reflex to larger volume changes; changes which before they produce appreciable buoyancy displacement, alter swimbladder pressure and appear to stimulate a pressure or tension reflex. The reflex

response in either case is appropriate fin movement which either maintains position or changes depth to return swimbladder volume and pressure toward poised, resting buoyancy. In these reflexes the fish is sensitive to at least 1.0 cm H_2O differential pressure in the swimbladder, and to buoyancy alteration of about 1.0% from neutral bladder volume. The swimbladder could accommodate 100% increase in volume, near neutral buoyancy, with only about 3–10 cm H_2O pressure increase. The behavior in swimming to compensate buoyancy changes, in a limited body of water, is stereotyped.

Resting bladder pressure is about 3.6 cm H_2O above ambient pressure, and there is reflex compression of swimbladder gas through a range of about 3.0 cm H_2O. This reflex appears to be effected through body musculature investing the swimbladder and its lateral lobes, though intrinsic muscles may be involved. A special response to small, rapidly imposed, negative pressure in the swimbladder, when the fish is near neutral buoyancy, is a mass reflex which is described as a *yawn*. This appears analogous in appendages, trunk, and buccal musculature activity to the mammalian yawn. Other behavioral patterns occurred which involved fins and trunk as the fish neared poised, resting buoyancy; these resembled nestling movements of birds and mammals. Yawning, nestling, and visual or vibratory stimulation were accompanied by discrete pressure changes in the bladder.

The muscular control of swimbladder pressure and volume is considered a fine adjustment over the coarser, longer range acclimatization by secretion and absorption of bladder gas. Such control would have adaptive value in maintenance of immobilization at rest and eluding predators. An absorption rate of 0.25 ml/hr. (S.D. 0.03) and a secretion rate of 0.10 ml/hr. were found.

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MORPHOGENESIS AND METABOLISM OF AMPHIBIAN LARVAE AFTER EXCISION OF HEART

III. EFFECT OF SODIUM AZIDE ON RESPIRATORY METABOLISM OF HEARTLESS LARVAE OF RANA PIPIENS [1]

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FOUR FIGURES

Previous publications (Kemp, '53; Kemp and Quinn, '54) have described some of the anomalies in the morphogenesis of larvae of *Rana pipiens* or *Amblystoma punctatum* made circulationless by excision of the heart. Both gross and microscopic observations on heartless tadpoles of the frog reveal a considerable departure from normal development; yet the animals may survive and continue differentiation up to two weeks after cardiectomy. Heartless tadpoles become different from the normal within a day after excision of the heart and develop a characteristic syndrome which includes: edema and a consequent broadening of the head, collapse of the vitreous chamber of the eye, failure of the intestine to coil, ballooning of the dorsal mesentery, and retarded utilization of yolk.

Circulating blood performs a variety of functions (Kemp, '51a). It is necessary for the maintenance of normal osmotic equilibrium (Rappaport, Jr., '55); it transports oxygen to the internal tissues and thereby facilitates accelerated respiratory metabolism; it may transport other substances, such as hormones, which stimulate metabolism. Exploratory measure-

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ments of the oxygen consumption of heartless larvae both of *Rana pipiens* and *Amblystoma punctatum* (Kemp, '51b, '55; Kemp and Quinn, '51) showed that the respiratory rate continues to rise for a few days following excision of the heart at a stage shortly after establishment of circulation. Eventually the rate levels off at about half the maximum rate achieved by normal animals before they reach the feeding stage. Details of the changing metabolism of heartless frog tadpoles as compared with controls will be presented here, together with data on the effects of the respiratory inhibitor, sodium azide. The immediate object in studying the effects of azide was to reveal any differential susceptibility of heartless and normal animals. If such differentials could be demonstrated, we reasoned that they might help to characterize qualitatively the increment of metabolism which is mediated by circulating blood.

MATERIAL AND METHODS

Embryos of *Rana pipiens* were obtained by artificial ovulation and fertilization of eggs of frogs obtained from a commercial supplier in Wisconsin. Reported data were obtained only from animals raised at room temperature, which was usually $\pm$ 23°C from November to April. Embryos developed in 12 $\times$ 18-inch enameled pans in about one inch of water up to the stage of operation. We used pond water or water from an aquarium when available but during the winter months we usually used tap water conditioned by bubbling air through it for several days with the aid of "air stones" obtained from a supplier of accessories for aquaria. Hearts were removed from larvae at Shumway stage 20 when circulation of blood through the gills is clearly apparent. This stage was generally attained by the fifth day after fertilization. Heartless larvae were cultured in Holtfreter's solution for an hour or two after operation, then transferred to 1/5 Holtfreter's solution. After the first day, heartless animals were cultured in pond water. Following this or a similar regimen is important for survival of heartless animals more than a day or two.

Respiratory measurements were made with standard size (16 ml) Warburg respirometers. For measurements on the day of cardiectomy, animals were introduced into the Warburg flasks in 1/5 Holtfreter's solution; thereafter they were introduced in conditioned water. A special funnel made from 11 mm glass tubing, bent so that the lower end would lead to the space outside the center well, was used for introducing into the flasks a volume of 3 ml, including 10 animals. The center well received 0.2 ml of 20% KOH when oxygen consumption was being measured. The "direct method" (Umbreit, Burris and Stauffer, '49) was used for calculating output of CO_2. In order to learn the pattern of QO_2 values over a period of several days from the time of cardiectomy, animals in some experiments were saved and their respiration measured daily. After each day's run they were emptied from the flasks into a large volume of tap water, then returned to pond water or conditioned water for continued culture. Saving animals necessitated calculation of results on the basis of μl O_2/animal/hour.

Stock solutions of 10^{-2} M or 10^{-3} M sodium azide were made up in pond water or conditioned tap water and diluted to the proper concentration just before use, if the desired concentration differed from that of the stock solution. Inhibitory concentrations ranging from 10^{-4} M to 10^{-3} M were routinely used after a series of trial runs to determine the effects of azide on rates of development, heart beat and respiration. In some experiments animals were permitted to develop continuously in 10^{-4} M azide and their respiration measured daily. In other experiments, however, animals were kept in water until a desired stage was reached; then they were transferred to azide solution. There was little change in the pH of unbuffered azide solutions containing animals over a period of 4 days; but in view of Keilin's ('36) and Armstrong and Fisher's ('40) demonstrations that pH affects inhibition by azide, we controlled the pH in some experiments by means of phosphate buffer.

RESULTS

Respiratory rates of normal and heartless larvae

Measurements of the "normal" consumption of oxygen and of CO_2 production of a group of tadpoles reared in pond water are recorded in table 1 and graphed in figure 1. Curves such as those of figure 1 are reproducible, although day-to-day fluctuations differ for different groups of animals. Following excision of the heart on day 0, the respiration of heartless animals increases for several days, but at a slower rate than that of normal animals. In the experiment reported here,

TABLE 1

Oxygen consumption, CO_2 production and R.Q. of a group of animals reared in pond water. Measurements made on the same animals at daily intervals for 6 days

	NORMAL ANIMALS			HEARTLESS ANIMALS		
Day	O_2 in μl/ animal/hr.	CO_2 in μl/ animal/hr.	R. Q.	O_2 in μl/ animal/hr.	CO_2 in μl/ animal/hr.	R. Q.
0	2.15	1.35	.63	2.03	1.37	.67
1	3.12	1.60	.51	2.82	2.23	.79
2	3.80	2.58	.68	3.19	2.72	.85
3	4.97	3.47	.70	2.63	1.96	.75
4	6.02	4.25	.70	3.76	2.71	.72
5	5.69	3.87	.68	3.73	3.29	.88

oxygen consumption for the normal animals rose from a value of 2.15 µl/animal/hour on day 0 to 6.02 on day 4, then started to decline, probably because the animals were running low in their endogenous substrates for release of energy. During the same period oxygen consumption in heartless animals increased from 2.03 µl/animal/hour on day 0 to a high of 3.76 on day 4. Production of CO_2 roughly paralleled oxygen consumption. Respiratory quotients were slightly higher for heartless animals every day, but the slight differences for days 0 and 4 are probably not significant. Whether the observed differences reflect the utilization of different kinds or different concentrations of substrates in normal and heartless animals is not known at present.

Effect of feeding normal larvae

The effect of feeding animals with strained spinach after they have passed the peak of the pre-feeding respiratory rate is illustrated in figure 2. Measurements of oxygen consumption were begun on the fifth day of development (day 0). It

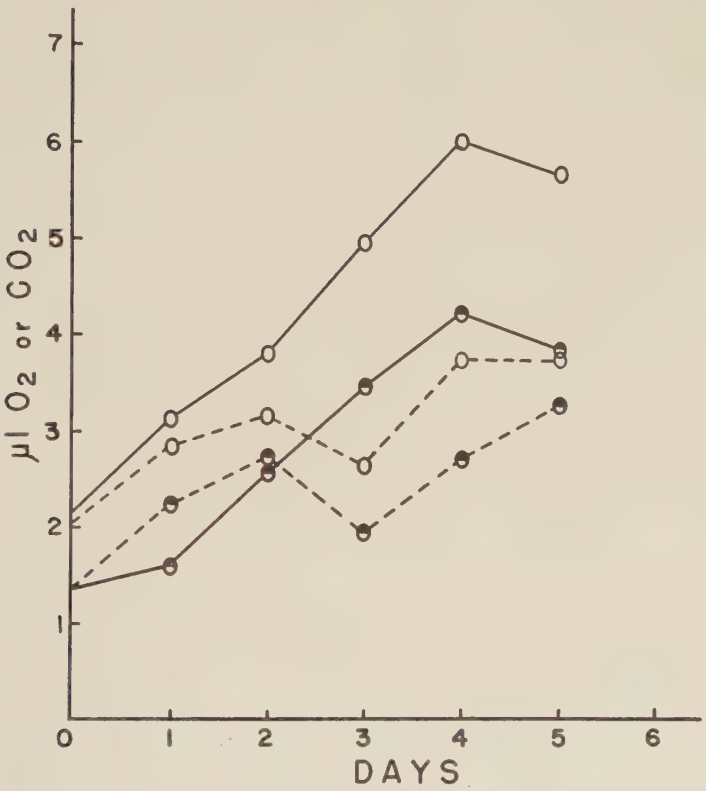

Fig. 1 Respiration of normal (solid lines) and heartless (broken lines) tadpoles, measured at daily intervals from the time of cardiectomy at stage 20 (day 0). Open circles represent μl O_2/animal/hour. Half-closed circles represent μl CO_2/animal/hour.

can be seen that respiration rose continuously to day 4, then began to fall off. On day 5 some animals were fed strained spinach, and another group were cultured without added food. Respiration in fed animals rose dramatically to parallel the

rate of increase observed between days 3 and 4, but it continued to decline in unfed animals.

Referring to table 1, one observes (column 5) that the maximum oxygen consumption by heartless animals, 3.76 µl on day 4, was approximately equalled in normal animals (column

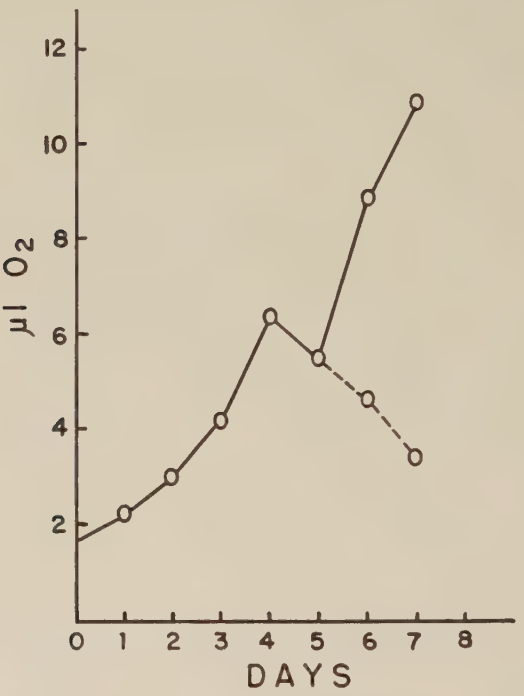

Fig. 2 Effect of feeding on oxygen consumption. Solid line up to day 5 represents normal respiratory rate in µl O_2/animal/hour. On day 5, animals were divided into two groups, one of which was fed (continuing solid line) and the other not fed (broken line).

2) on day 2. In figure 2 we see that the rate in unfed normal animals returned to about the same value on day 7. It seems probable that the three nearly equivalent values for QO_2 were obtained from animals in considerably different physiological states. Heartless animals on day 4 were utilizing yolk relatively slowly, and enzyme synthesis had probably leveled off at an equilibrium value. Normal animals on day 2 were rapidly

digesting yolk and probably synthesizing respiratory enzymes rapidly; furthermore, circulating blood could speed the transport of oxygen and other metabolites. Normal animals by day 7 had used up most of their reserves of yolk and evidently were deficient in endogenous substrates for the release of energy.

To test the hypothesis that added substrate accounted for the rise in respiratory rate in normal animals after feeding with spinach, we conducted a series of experiments in which respiration of animals in 0.1% or 1% glucose was measured. Results of an experiment lasting 7 days are shown in table 2. Measurements were begun on the sixth day of development,

TABLE 2

Effect of glucose on oxygen consumption of normal tadpoles reared in conditioned water

DAY	μL/ANIMAL/ HR. IN WATER	μL/ANIMAL/ HR. IN 0.1% GLUCOSE	PER CENT INCREASE	μL/ANIMAL/ HR. IN 1% GLUCOSE	PER CENT INCREASE
1	2.14	2.36	5.6	2.29	7.0
2	3.17	3.27	3.1	3.26	2.8
3	4.45	4.67	4.9	5.27	18.4
4	6.96	7.39	6.2	7.58	8.9
7	4.73	5.39	13.9	5.70	20.5

corresponding to day 1 of the experiment illustrated by figure 1. During days 1 to 4 of the experiment, we expected that the normal respiratory rate would be rising; but by day 7 we knew it would have declined. The values shown in table 2 are averages for two vessels each containing 10 animals. Different animals from the same original batch were used each day. Although the glucose solutions were not sterilized, the possibility that contaminating bacteria or molds might affect our results was unlikely, since fresh glucose solutions were prepared each day.

Table 2 indicates that in 0.1% glucose there was relatively little acceleration of respiration during the first 4 days of the experiment but on day 7 the per cent of increase was up to 13.9%. Acceleration of respiration was greater in 1% glucose on every day except day 2. The enhancement of the rate rose to

18.4% on day 3 and to 20.5% on day 7. From this experiment it appears that the substrate glucose when added to the culture fluid does not cause much change in respiratory rate during the early days of larval differentiation when conversion of

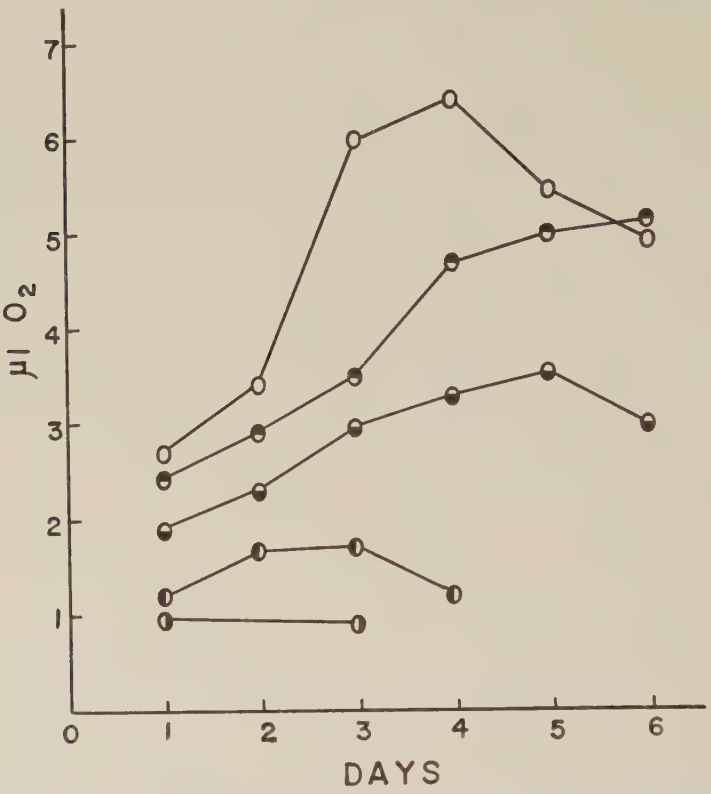

Fig. 3 Effect of concentration of sodium azide on respiration of normal tadpoles. Respiration calculated at daily intervals as μl O_2/animal/hour in water (open circles), 10^{-4} M sodium azide (upper half of circles closed), 2×10^{-4} M azide (lower half of circles closed), 5×10^{-4} M azide (left half of circles closed), and 10^{-3} M azide (right half of circles closed). Animals cultured in water or azide solutions continuously from day 0 until day of measurement. Respiration measured at daily intervals beginning on day 1.

yolk to protoplasm is proceeding rapidly. When yolk reserves run low and the normal respiratory rate is declining, however, as on day 7, added glucose has a pronounced stimulatory effect. There is evidence too that glucose may stimulate respiration

significantly on day 3, one day before the peak normal respiratory rate has been reached. Results of other similar experiments with glucose are consistent with those in the experiment reported, but we have not tested the effects of adding other substrates.

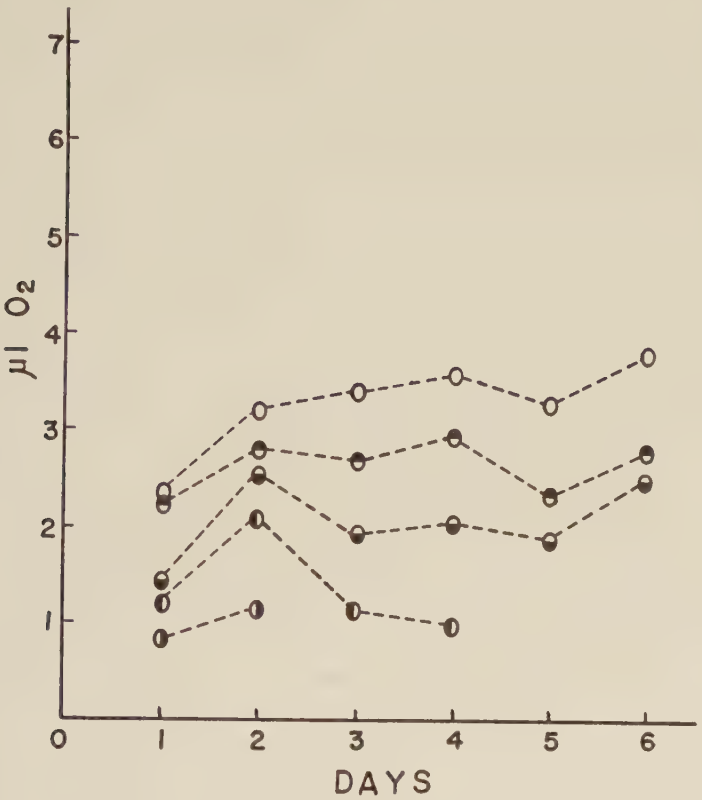

Fig. 4 Effect of concentration of sodium azide on respiration of heartless tadpoles. Basis of calculation and concentrations of azide same as for figure 3.

Inhibition of respiration by sodium azide

Effect of concentration. In order to determine concentrations which would be useful in demonstrating differential susceptibility of normal and heartless animals, a series of animals at stage 20 were placed in solutions of azide in pond water ranging in concentration from 10^{-4} M to 10^{-3} M. In one group

of animals cultured continuously in 10^{-4} M azide after reaching stage 20, the rate of heart beat was inhibited to a level 25% below the normal rate of 138 beats per minute on the first day after beginning exposure. On subsequent days inhibition diminished somewhat but was still 19% on day 5. Inhibited animals were definitely smaller than normal animals on day 5; their intestines had 1½ coils compared with the 2½ coils usually found in normal animals of this age; and considerable yolk remained in the intestinal epithelial cells. Inhibited animals, however, seemed no less responsive to tactile stimuli than normal animals.

A concentration of 2×10^{-4} M azide inhibited development and rate of heart beat still more. By 1 day after exposure the heart rate was inhibited 32% and by 5 days, 27%. The intestine by 5 days had but 1½ coils and retained even more yolk than in animals exposed to 10^{-4} M azide. Another observation was that the intestines in these animals were collapsed like flat tires, in contrast to the inflated tube-like intestines of normal animals. Evidently azide was somehow affecting osmoregulation of the intestine. Increasing the concentration of azide to 5×10^{-4} M resulted in 42% inhibition of the rate of heart beat by one day after exposure and 55% on the second day. Circulation had ceased by the fifth day. No operculum developed at this concentration of azide and there was no elongation or coiling of the intestine. Circulation in animals exposed to 10^{-3} M azide was completely arrested by the first day, and all animals were dead by the second day after exposure.

Respiratory measurements begun approximately one day after exposure of a group of animals to various concentrations of sodium azide are graphed in figures 3 and 4. The graphs demonstrate the increasing effect of the inhibitor in concentrations from 10^{-4} M to 10^{-3} M both among normal and heartless animals. The graphs show also that among normal animals there was a general increase in rate of respiration on successive days even in the presence of the inhibitor, whereas among heartless animals the respiratory rates tended to level off after the second day. For about three days the respiratory rate of

normal animals in 10^{-4} M azide was lowered approximately to the level of the rate of heartless animals in pond water. Thereafter the susceptibility of normal animals in 10^{-4} M azide decreased, and they respired at a higher rate than heartless animals in pond water.

Effect of time. Since sodium azide does not exert its effect immediately, the increasing effect with time was measured for both normal and heartless animals in several different concentrations of inhibitor and for various spans of time. It required several hours to detect appreciable inhibition by concentrations of 10^{-4} M and 2×10^{-4} azide, but concentrations of 5×10^{-4} M and 10^{-3} M inhibited considerably within an hour after exposure. Data for an experiment with 5×10^{-4} M azide are recorded in table 3.

Before this experiment was begun on day 0, enough tadpoles at stage 20 were cardiectomized so that 40 could be used each day for 5 days. On each day 10 heartless animals were placed in each of two Warburg vessels containing pond water. Ten additional animals were placed in each of two vessels containing 5×10^{-4} M azide. Forty normal animals were similarly distributed in 4 other vessels — 20 animals in pond water, 20 in 10^{-4} M azide. As soon as the vessels and manometers could be assembled and the vessels equilibrated, respirometry was begun. Usually about two hours had elapsed from the time of initial exposure of animals to azide until the end of the first hour of measurements. Only the measurements for the first, fourth and eighth hours are shown in table 3. New animals which had been developing at room temperature were used each day.

On day 0 of the experiment there was very little effect of the inhibitor after one hour (table 3, columns 5 and 8). By 4 hours, inhibition was up to 21.6% in normal animals but was a negative 65%, meaning that there was acceleration of respiration, in heartless animals. By 8 hours on day 0 inhibition of normal animals was 56%, and acceleration of respiration in heartless animals was down to 12%. On subsequent days inhibition by the end of the first hour was already a large fraction of the

inhibition after 8 hours, both in normal and heartless animals. It seems significant that inhibition of heartless animals after 8 hours on days 2 and 3 was considerably less than inhibition of normal animals at the same times. By day 4 susceptibility to azide after 8 hours was about the same in normal and heartless animals. Perhaps the metabolism of normal animals had begun to slacken by this time.

TABLE 3

Effect of time on inhibition of respiration of normal and heartless tadpoles in 5×10^{-4} M NaN_3

DAY	HOURS AFTER BEGINNING OF RESPIROMETRY	$\mu L/O_2$/ANIMAL/HOUR		% INHIBITION OF NORMAL ANIMALS	$\mu L/O_2$/ANIMAL/HOUR		% INHIBITION OF HEARTLESS ANIMALS
		Normal animals in H_2O	Normal in 5×10^{-4} M NaN_3		Heartless animals in H_2O	Heartless in 5×10^{-4} M NaN_3	
0	1	2.31	2.22	3.9	2.04	2.09	— 2.0
	4	3.42	2.68	21.6	1.58	2.60	— 65.0
	8	2.93	1.29	56.0	2.25	2.51	— 12.0
1	1	3.74	2.01	46.3	3.28	1.56	52.4
	4	3.33	1.59	52.3	2.82	0.82	70.9
	8	3.92	1.42	63.8	3.04	1.13	62.8
2	1	3.89	1.86	52.2	3.28	2.94	10.4
	4	4.35	2.46	43.2	3.21	2.10	34.7
	8	4.80	2.21	54.1	3.25	2.20	32.3
3	1	5.89	2.86	51.4	3.43	2.20	35.9
	4	6.06	2.25	62.9	3.65	2.42	33.7
	8	5.63	1.93	65.7	3.24	1.99	38.6
4	1	5.37	3.21	40.2	2.80	2.06	26.1
	4	5.64	3.16	44.1	3.58	2.06	42.5
	8	5.60	2.69	52.0	3.22	1.51	53.1

Effect of azide on animals exposed at different ages. In order to investigate further the factor of age in susceptibility to azide, we conducted an experiment in which animals at daily intervals were placed in 2×10^{-4} M azide buffered with M/500 phosphate buffer to pH 7.4–7.5 and their respiration measured one day after exposure. Animals on day 0 were at stage 20, and new animals were used each day. Time of exposure to azide before respirometry was approximately the same for all groups. Results are recorded in table 4.

There is no doubt that susceptibility of normal animals increases with age. Inhibition was 19.6% and 17.9% respectively for animals exposed to azide on days 0 and 1. Coinciding with the period of accelerating metabolism on days 2 and 3, however, there was a sudden rise in susceptibility to azide. Measurements on days 3 and 4 for animals exposed on days 2 and 3 revealed inhibitions of 43.3% and 40.9%. After this rise in susceptibility there was a decline, as indicated by the reduced

TABLE 4

DAY	$\mu L/O_2/ANIMAL/HOUR$		% INHIBITION OF NORMAL	$\mu L/O_2/ANIMAL/HOUR$		% INHIBITION OF HEARTLESS
	Normal in H_2O	Normal in 2×10^{-4} azide		Heartless in H_2O	Heartless in 2×10^{-4} azide	
0	2.59					
1	3.06	2.46	19.6	2.34	2.22	5.1
2	4.97	4.08	17.9	3.14	2.83	9.9
3	5.27	2.99	43.3	3.24	3.12	3.7
4	6.06	3.58	40.9	2.95	3.23	— 9.5
5	5.81	—	—			
6	5.47	4.78	12.6			
7	5.69	5.06	11.1			
8	4.19	3.71	11.5			
9	4.37	3.58	18.1			

percentages of inhibition recorded on days 6 to 9. In heartless animals the inhibitory effect of azide was always considerably less than for normal animals. Indeed on day 4 the heartless animals exposed on day 3 showed about 10% acceleration of respiration.

DISCUSSION

Interpretation of our results on the differences in respiratory metabolism of normal and heartless tadpoles should be based on an intimate knowledge of the metabolism of the normal embryo. Papers by Gregg and Ballentine, '46; Boell, '45, '48; Gregg, '48; Barth and Barth, '51; Krugelis, '50; Kutsky, '50; Krugelis, Nicholas and Vosgian, '52; Løvtrup, '53c; Cohen, '54, give details on the quantitative changes of particular substances during amphibian development, but we have

only a general idea of how the metabolism of these substances is related to total respiration. Boell ('55) states that increases in respiratory metabolism probably reflect "the transformation of yolk into protoplasm," but Løvtrup ('53a) points out that "yolk" in this general sense "means any kind of reserve material present in the fertilized egg." In the restricted sense "yolk" refers to the yolk platelets composed of phospho-lipoprotein.

If one plots the rate of oxygen consumption of amphibian embryos from the time of fertilization, one notes a constant rate of increase up to a point of inflection (Atlas, '38; Barnes, '44; Moog, '44; Spiegelman and Steinbach, '45; Boell, '45, '55) or a plateau (Løvtrup, '53a, '53b; Tuft, '53) or gently rising slope (Ten Cate, '56), followed by a changed but again constant rate of increase reaching a maximum at about the time feeding begins. Løvtrup ('53a) states that "it is impossible to correlate the shape of the respiratory curves with other observed phenomena." He demonstrates, however, that the period of constant or slow rise of respiration between the two periods of logarithmic increase lies in the late neurula and the first tailbud stages. It appears from his curves that the beginning of the rise in respiration which follows neurulation coincides with the decline of consumption of carbohydrate and the beginning of fat consumption. Boell ('45) has attributed the changing slope of the respiratory curve during or after neurulation (1) to some change in the transformation of "yolk" into embryo or (2) to an altered surface-volume ratio. Tuft ('53) believes that the rise in respiration after neurulation is a reflection of the acceleration of cellular differentiation beginning then.

Measurements which have been made during the period of establishment of circulation in amphibians (heart begins to beat at Shumway stage 19 for *Rana pipiens,* Harrison stage 34 for *Amblystoma punctatum*) do not show any abrupt change in rate of respiratory increase at that time (Wills, '36; Atlas, '38; Fischer and Hartwig, '38; Hopkins and Handford, '43; Boell, '45; Spiegelman and Steinbach, '45; Barth, '46;

Løvtrup, '53a; Ten Cate, '56). Amberson and Armstrong ('33), however, reported data which indicate a decided inflection in respiratory rate coinciding with the establishment of circulation in *Fundulus heteroclitus*. Our own studies have been concerned chiefly with a period beginning at Shumway stage 20, about a day after the establishment of circulation, and extending to the time when the animals are capable of feeding. It is true that circulation of blood does give a normal animal an advantage which gradually results in a higher rate of increase of respiration than that of a heartless animal; but it is probably also true that circulation *per se* does not result in respiratory increase. More plausible is the hypothesis that circulating blood gradually speeds up the rate of utilization of yolk with a resultant increased synthesis of metabolites, including respiratory enzymes such as cytochrome oxidase and succinoxidase (Boell, '45, '48, '55). Boell ('45) has shown that the quantity of cytochrome oxidase in *Amblystoma punctatum* starts to climb shortly before Harrison stage 34, when the heart begins to beat, and continues rising to a maximum at stage 46. In unfed animals the enzyme level then remains constant or decreases somewhat. From a comparison of curves for increase of cytochrome oxidase and for respiratory increase, however, Boell came to the conclusion that absolute rate of oxygen consumption is controlled by some factor other than the concentration of cytochrome oxidase. In a later discussion ('55) he mentions some of the possible limiting factors such as amount of substrate, rate of mobilization of substrate, affinity between enzyme and substrate, or limiting concentrations of essential intermediates.

Coupling of glycolysis with phosphorylation is one factor controlling respiratory rate, for it is well known (McElroy, '47) that certain agents which uncouple phosphorylation and glycolysis, e.g., phenyl urethane, chloral hydrate or chloretone, phenobarbital and amytal, dinitrophenol and various substituted phenols, azide, and gramicidin, may in appropriate concentrations stimulate oxygen consumption.

Amberson and Armstrong ('33), working with Fundulus, and Boell ('35), working with the grasshopper, have supported Needham's ('31, '42) conclusion that the sequence of energy sources for embryonic differentiation in many groups of animals is carbohydrate, protein and fat. For the salamander, *Amblystoma mexicanum,* however, Løvtrup ('53a, '53b) has concluded that the order of utilization of food reserves is carbohydrate, fat and protein. Carbohydrate utilization in this species reaches a maximum during neurulation, whereupon fat consumption begins to rise. Protein combustion begins shortly before the maximum for fat consumption is reached (days 17–19 in *A. mexicanum*). Utilization of protein becomes maximal at 22 days after most of the yolk has been digested. If there is a comparable sequence in *Rana pipiens,* it is probable that our tadpoles were well within the period of fat utilization when the experiments were begun at stage 20.

The R.Q. values we obtained for normal animals, approximately 0.70 for days 2–5 (table 1), agree with Atlas' ('38) results for *Rana pipiens* and also with those of Amberson and Armstrong ('33) for *Fundulus heteroclitus.* Since bound CO_2 was not measured, however, it is probable that our R.Q. values are too low. Quoted values of 0.71 for pure fat and 0.80 for protein combustion (Harrow, '54) are helpful guides, but R.Q. values can be easily misinterpreted (Boell, '55). Fiske and Boyden ('26), for example, reported that the R.Q. for protein combustion could be 1.0, 0.8, or 0.7 depending on whether the end product were ammonia, urea, or uric acid. The generally higher R.Q. values (.72–.88 on days 2–5) in our heartless animals probably reflect a difference from the metabolism of normal animals, although we have insufficient information to warrant speculation on the nature of the difference. Another instance of differing R.Q. values was reported by Barth ('46), who found that blocked hybrid frog embryos have higher respiratory quotients than normal post-gastrular embryos.

Turning to the question of susceptibility to sodium azide, we should like to consider the meaning of the observed differences in normal animals of different ages and in heartless

animals compared with normal controls. Barth and Barth ('54) have emphasized that permeability is one important problem encountered in studies with metabolic inhibitors of the frog egg. Since our animals were already swimming larvae and in most experiments were exposed overnight before measurements of respiration were begun, we assume that azide had ample time to penetrate.

McElroy's ('47) review on the effects of narcotics on cellular activity calls attention to work by Stannard ('39a, '39b), Armstrong and Fisher ('40), Fisher and Stern ('42), Fisher and Henry ('44) and Ormsbee and Fisher ('44) giving evidence for two qualitatively different kinds of metabolism with respect to sensitivity to narcotic agents. Stannard's theory that "maintenance" and "activity" metabolism are qualitatively different has been largely abandoned. McElroy ('47) pointed out though that the greater sensitivity of the so-called "activity" systems appears to be related to the fact that they involve synthetic reactions, which are customarily endergonic and therefore coupled with exergonic, energy-yielding reactions.

Spiegelman, Kamen and Sussman ('48) have presented evidence indicating that azide uncouples glycolysis from oxidative phosphorylation. Azide is also known to inhibit the cytochrome oxidase system (Keilin, '36; Barth, '46; Spiegelman, Kamen and Sussman, '48) and hence interferes with oxygen uptake during aerobic metabolism. These facts assume significance in relation to the knowledge (McElroy, '47) that oxygen consuming reactions are generally from two sources: (1) those associated with glycolysis and (2) those associated with the subsequent oxidation of various intermediate compounds.

Normal animals exposed to azide on days 2 and 3 of our experiments showed the greatest susceptibility to the inhibitor. Differentiation, involving intense synthetic activity, was proceeding rapidly on these days, and we may hypothesize that tadpoles had become relatively rich in susceptible compounds such as cytochrome oxidase and phosphopherase. Conversely, we may hypothesize that the concentrations of susceptible com-

pounds were relatively low in the less susceptible normal animals and in heartless animals. We can not readily explain though how differing concentrations of susceptible enzymes could alone explain differential susceptibility to azide. It seems necessary to invoke the theory that the susceptible compounds are differentially affected by azide and that the relative concentrations of these compounds fluctuate during development. For example, if azide inhibits phosphopherase more strongly than cytochrome oxidase, then we would expect greater inhibition of respiration during periods when the relative concentration of phosphopherase was high. It is possible that these hypothetical conditions come close to explaining the high susceptibility of normal animals on days 2 and 3 of our experiments. For a more precise analysis of the respiratory metabolism of heartless and normal tadpoles we need information on the fluctuating concentrations of compounds known to be important in the control of respiratory rate. We also need information on the relative susceptibility of rate limiting enzymes to azide or other more specific inhibitors.

SUMMARY

1. Respiration of a group of frog tadpoles cardiectomized at stage 20 (day 0) was compared with that of normal animals. For 4 days the respiratory rate continued to increase and reached a high of 6.02 μl O_2/animal/hour in normal animals while reaching 3.76 μl/animal/hour in heartless animals. The rate declined in normal animals on the fifth day but remained at the 4-day level in heartless animals. Respiratory quotients were about 0.7 for normal animals over the period measured but were generally higher (up to 0.88 on day 5) in heartless tadpoles.

2. Oxygen consumption of normal animals fed spinach on day 5 resumed a steady rise, whereas respiration continued to decline in unfed animals. Glucose added to the culture medium did not influence the respiratory rate much until day 3. In 1% glucose on day 7 the O_2 consumption was 20% greater than for animals in pond water.

3. The inhibitory effect of sodium azide increased directly with concentration. Normal animals were generally more susceptible than heartless animals to concentrations ranging from 10^{-4} M to 10^{-3} M azide.

4. The effect of time on inhibition by 5×10^{-4} M azide was investigated for both normal and heartless animals. On the day of cardiectomy, inhibition increased in normal animals from 3.9% after one hour to 21.6% after 4 hours to 56.0% after 8 hours. In heartless animals on this day, respiration was actually accelerated. On days 1 to 4, inhibition in normal animals after one hour was already about 40–50%, and inhibition after 8 hours increased only to 50–65%. In heartless animals on days 1 to 4, inhibition after one hour varied from about 10–50% and after 8 hours from about 30–60%.

5. Differences in the susceptibility of animals exposed to 2×10^{-4} M azide solutions at different ages were detected. Normal animals exposed on days 2 and 3 after the stage of cardiectomy were inhibited by about 40% while those exposed on days 0 and 1 (respiration measured on days 1 and 2) or on days 5 to 8 (respiration measured on days 6 to 9) were inhibited by about 10–20%. Heartless animals exposed on days 0, 1, 2 and 3 were never inhibited more than 10%.

6. The results are interpreted as indicating that normal tadpoles during the period of greatest susceptibility to azide are richest in azide-susceptible compounds such as cytochrome oxidase and phosphopherase. Heartless animals and normal animals during periods of low susceptibility probably have relatively low concentrations of susceptible enzymes or their substrates. If azide selectively inhibits phosphopherase or other rate-limiting compounds important for synthetic reactions, the increment of respiration which increases during high synthetic activity may be selectively curtailed and respiration brought back more nearly to a "maintenance" level.

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FLAVIN ENZYMES IN LIVER AND KIDNEY OF RATS FROM BIRTH TO WEANING [1]

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TWO FIGURES

This is a report of the changes during the first weeks of life of the rat in the concentration of three oxidases of kidney and liver. The study was made practicable by the availability of new analytical methods for these enzymes which require but a few milligrams of tissue per analysis (Burch et al., '56). Consequently, a single kidney weighing 25 mg from a newborn rat could be analyzed in duplicate for the enzymes, xanthine oxidase, and D-amino acid oxidase, as well as protein and total riboflavin. In addition the method for xanthine oxidase is sufficiently sensitive to measure the low level of activity in liver at birth which escaped detection by earlier methods (Richert et al., '49).

Striking increases were found for the three enzymes from birth to weaning. It was also observed that the livers of the post partum mothers are low in D-amino acid oxidase activity and that the livers of adult females in general contain only about half as much glycolic acid oxidase as the livers of adult male rats.

EXPERIMENTAL

The rats were Sprague-Dawley strain from Holtzman Rat Co., Madison, Wis. Pregnant rats were shipped to this laboratory several days before term. Most analyses were made with litters from mothers fed Purina Dog Chow after arrival. A

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second group of pregnant rats received a synthetic diet high in riboflavin (Burch et al., '56) during the last 10 days of gestation. No differences were found between the two groups, in respect to the substances measured, in the tissues of the mothers or the offspring.

At each test period one or two rats were taken from each of 5 litters. Liver and kidney were analyzed from 21 rats within 12 hours of birth; from 12 rats at 4 days, 7 at 9 days, 6 at 12 days, 7 at 14 days and 4 at 21 days after birth. The organs of maternal rats, sacrificed while under ether anesthesia by bleeding from the heart as completely as possible, were analyzed immediately post partum or after nursing for 21 days. Young rats were decapitated and the kidneys and liver were rapidly dissected and weighed. Homogenates were prepared either at once in 0.02 M phosphate buffer pH 8.3 at 0° or after storage of the tissues in liquid nitrogen for 24 hours.

Total riboflavin was measured fluorometrically after acid hydrolysis of tissue extracts (Burch, '57) and protein was determined colorimetrically (Lowry et al., '51). Xanthine oxidase activity was measured by the rate of oxidation of 2-amino-4-hydroxypteridine to fluorescent isoxanthopterin (Burch et al., '56). This method completely avoids the usual troubles from tissue blanks. D-amino acid oxidase and glycolic acid oxidase activities were determined by spectrophotometric measurement of the 3-hydrazinoquinoline derivatives of α-keto acids formed when these enzymes oxidize D-alanine and glycolic acid respectively.

RESULTS

The riboflavin and enzyme values have been calculated on the basis of protein because of the well known changes in tissue protein concentration during growth (table 1, figs. 1 and 2).

From birth to 21 days (table 1) the concentration of hepatic xanthine oxidase, D-amino and glycolic acid oxidases increased 12-, 5- and 5-fold respectively. Riboflavin and protein at 21 days were respectively 160 and 129% of the newborn levels.

By 21 days of age protein, D-amino acid oxidase, and glycolic acid oxidase of the liver had risen to about the average values for adult males and females. Xanthine oxidase and riboflavin were still 40 and 16% below the adult level. The

TABLE 1

Flavin oxidases, riboflavin, and protein in liver and kidney of rats of various ages

Enzyme activities are expressed as millimoles of substrate oxidized per kilo of protein per hour. The figures in italics are the standard errors of the mean.

	PROTEIN	RIBOFLAVIN	D-AMINO [3] ACID OX.	GLYCOLIC ACID OX.	XANTHINE OXIDASE
	gm/kg	*mg/kg protein*	*mM KpHr.*	*mM KpHr.*	*mM KpHr.*
Liver					
Newborn (21) [2]	138	88	158	266	3
	2	*2*	*7*	*11*	*1*
21 day old (4)	178	140	850	1239	35
	1	*2*	*65*	*22*	*2*
Adult post partum (5)	195	138	403	671	50
	7	*5*	*57*	*28*	*3*
Adult nursing 21 days (2)	201	135	906	716	48
Adult female, control (5)	178	161	922	759	54
	2	*1*	*124*	*31*	*2*
Adult male (5)	182	173	880	1430	66
	2	*3*	*101*	*22*	*3*
Kidney					
Newborn (21)	86	60	2,410		0.5
	2	*1*	*54*		*0.2*
21 day old (4)	142	132	12,100		6.6
	2	*3*	*450*		*0.2*
Adult post partum (5)	135	182	9,150		38
	4	*7*	*430*		*3*
Adult nursing 21 days (2)	163	154	10,500		23
Adult female control (5)	165	178	10,630		31
	4	*2*	*100*		*1*
Adult male (5)	163	204	11,640		34
	3	*5*	*60*		*1*

[2] Numbers of animals analyzed are indicated. The average body weights at birth and 21 days were 6.2 and 44 gm respectively. The average liver weights at birth and 21 days were 250 and 1830 mg respectively; the average combined kidney weights were 49.4 and 503 mg.

[3] The substrate was 0.25 M D-alanine instead of 0.5 M which was used by Burch et al., ('56). The higher substrate level gives about 20% higher activity.

pattern of increase (fig. 1) differed among the substances measured. Riboflavin underwent a steady increase toward the adult level during the 21 day period. Xanthine oxidase and D-amino acid oxidase increased at nearly parallel rates, with the greatest increase in the third week of life. Glycolic acid

oxidase nearly doubled in concentration between the ninth and twelfth days and then remained almost constant.

A surprising finding is that the D-amino acid oxidase activity of maternal liver immediately post partum was much lower than that of control adult female rats or of mothers nursing 21 days, whereas the other enzymes were not decreased. It

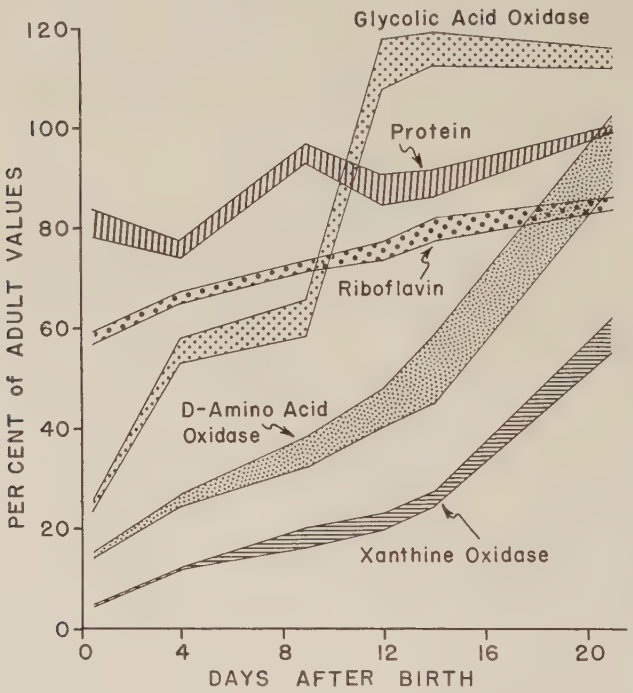

Fig. 1 Changes in protein, riboflavin and flavin enzyme activities of rat liver during postnatal development. Values are expressed as per cent of adult levels. Protein values are relative to wet weight. Riboflavin and enzyme activities are relative to protein. Thickness of the bands indicates ± one standard error.

will also be noted that glycolic acid oxidase is twice as high in the liver of the adult male, as in the adult female, and that there are statistically greater concentrations of riboflavin in liver and kidney of the male than in the same organs of the female. Studies of endocrine effects on riboflavin and on the hepatic content of the two enzymes would seem desirable.

In the kidney (table I, fig. 2), protein and riboflavin were relatively and absolutely lower at birth than in the case of the liver. The subsequent rise in riboflavin was approximately linear with age. As in liver the adult level was not attained by 21 days. D-amino acid oxidase increased about 5-fold in concentration, as in liver, but the increase was less uniform

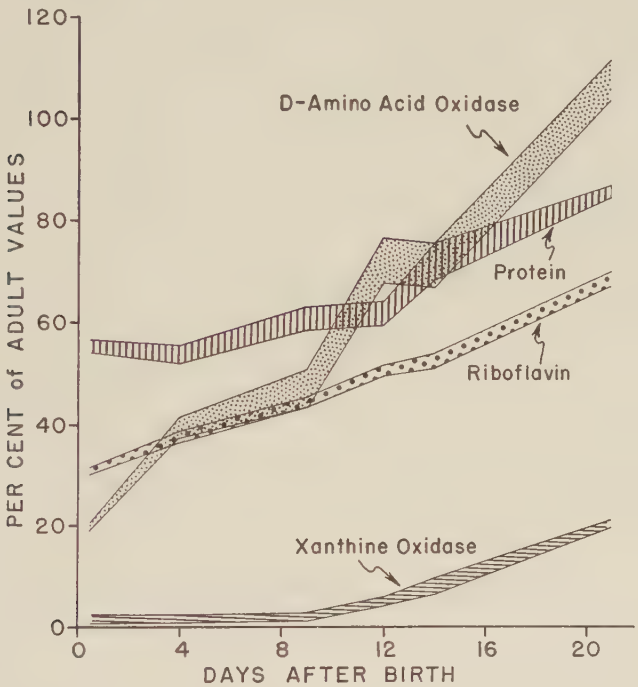

Fig. 2 Change in protein, riboflavin and flavin enzyme activities of rat kidney during postnatal development. Values are expressed as in figure 1. Thickness of the bands indicates ± one standard error.

with time. Xanthine oxidase was barely demonstrable for the first 9 days of life, and had only reached a fifth of the adult level by 21 days of age. Glycolic acid oxidase was not measured in kidney since its level has been found to be very low.

DISCUSSION

Rat liver has previously been analyzed from birth to weaning for D-amino acid oxidase (Kuriaki and Kensler, '54) and

at birth and weaning for xanthine oxidase (Richert et al., '49; Westerfeld and Richert, '49). These earlier results are in general agreement with those of the present report except that no xanthine oxidase was found in the newborn liver by the method of Westerfeld and Richert and the absolute levels of D-amino acid oxidase measured by Kuriaki and Kensler were only a quarter to a third of the present values. The low levels of both enzymes in the newborn would be particularly difficult to measure quantitatively by the oxygen consumption methods employed in earlier studies. No previous studies are available for rats under 21 days of age in regard to glycolic acid oxidase in liver or D-amino acid oxidase or xanthine oxidase in kidney. Previous reports of riboflavin in this age range are only fragmentary.

It is to be noted that in both kidney and liver there are not only differences in the rates of development of different enzymes, but the increase in total riboflavin is much less percentage-wise, than the increase of any of the enzymes measured. Since there is almost no free riboflavin in these tissues, it is likely that the riboflavin is equal to the sum of all the flavin enzymes present. If so, then some of the other flavin oxidative enzymes must be changing very little or perhaps decreasing (relative to protein) during maturation. In this connection, (Potter et al., '45) found that succinic dehydrogenase only doubled in activity in the liver from birth onwards based on wet weight of tissue (i. e., less than a 60% increase based on protein).

The present report is in agreement with the thesis of an increase in oxidative metabolism during post natal development. However, judging from the complex changes in the individual oxidative enzymes, the *character* of the oxidative pattern is changing greatly during this period.

In the case of the kidney, at least, it will be necessary to relate the enzyme changes to the developing histological structure before it is possible to assess changes in metabolic capacities of individual cell types.

Further discussion of these results seems unwarranted until values for a wider variety of oxidative as well as glycolytic enzymes are available.

SUMMARY

1. Xanthine oxidase, D-amino acid oxidase, glycolic acid oxidase, riboflavin and protein have been measured in liver and (except for glycolic acid oxidase) in kidney of newborn rats at intervals from birth to 21 days of age, in maternal rats immediately post partum and after nursing 21 days, and in normal adult males and females.

2. From birth to 21 days in liver riboflavin increased 60% and xanthine oxidase, D-amino acid and glycolic acid oxidases increased 12-, 5-, and 5-fold respectively (all relative to protein, which itself increased 29%). In kidney (relative to protein), riboflavin increased 120% and D-amino acid and xanthine oxidase activities increased 5- and 13-fold respectively. Protein itself increased 65 per cent.

3. At 21 days hepatic and renal xanthine oxidase values were only 60 and 20% respectively of those found in the adult, whereas D-amino acid oxidase and glycolic acid oxidase were near the adult levels.

4. D-amino acid oxidase was low in maternal liver at parturition.

5. Hepatic glycolic acid oxidase of the normal adult female was half of the level of the male.

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